University of Alberta

Development of a biological control for botrytis stem canker of greenhouse tomatoes caused by *Botrytis cinerea* Pers.

by

James Todd Calpas ©

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Abstract

Botrytis cinerea Pers. is a common fungal pathogen causing grey mould on a number of greenhouse ornamental and vegetable crops including greenhouse tomato (*Lycopersicon esculentum* Mill.). *Botrytis cinerea* infects tomato leaflets, petioles, stems, flowers and fruits.

The biological control potential of *Trichoderma* spp. is well recognized. The overall goal of this thesis research was to develop a biological control for *B. cinerea* on greenhouse tomato using a native strain of *Trichoderma* sp. from Alberta.

This study demonstrates some of the variability within *T. harzianum* and within *B. cinerea* using random amplified polymorphic DNA (RAPD) analysis. The variability present in *T. harzianum* indicated relationships between RAPD groupings and biological control potential. The better biological control strains grouped together and were generally distinct from the relatively poorer biological control strains.

RAPD analysis of *B. cinerea* clearly demonstrates the existence of geographic specialization in different strains of *B. cinerea* collected from across Alberta. Geographic specialization in *B. cinerea* has not been previously reported.

Two strains of *T. harzianum* (strains 7 and 29) from Alberta perform equally well as fungicides in reducing the length of lesions caused by *B. cinerea* as assessed using a greenhouse tomato petiole bioassay. As the tomato petiole bioassay was conducted in a greenhouse tomato crop managed to commercial production standards, these two biological strains of *T. harzianum* demonstrate excellent potential for commercialization.

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List of Abbreviations

ANOVA	analysis of variance
cv	cultivar
DNA	deoxyribonucleic acid
dNTPs	deoxyribonucleoside triphosphates
EDTA	ethylene diamine tetra acetic acid
GLM	general linear models
MXCOMP	matrix comparison
NTSYS	numerical taxonomy and multivariate analysis system
PCR	polymerase chain reaction
PDA	potato dextrose agar
RAPD	random amplified polymorphic DNA
RNA	ribonucleic acid
SAHN	sequential agglomerative hierarchical nested cluster analysis
SDS	sodium dodecyl sulphate
TBE	Tris borate
TE	Tris-EDTA
UPGMA	unweighted pair group method of arithmetic averages

Chapter 1

Literature review

1.1 Introduction

An expanding global population challenges agriculture to increase food production to meet the needs of humanity (Mannion 1998). From the mid 1960s to the mid 1990s agricultural production increased at a greater rate than population. This remarkable increase in agricultural production resulted from the successful application of science and technology to agriculture in areas including plant protection and environmental modification (Mannion 1998). The application of science is seen through the increased use of chemical fertilizers, and fungicides that have increased yield potential through optimizing growth and improved disease management. Plant breeding has also provided varieties with improved yield and disease resistance. Even with these improvements in yield potential and disease management, worldwide crop yield losses due to plant pathogens are still estimated to be almost 20% of the total production (Knight *et al.* 1997).

Agriculture is a significant agent of environmental change and has incorporated large areas of natural ecosystem into agricultural production (Mannion 1998). There is increasing concern about the ongoing loss of natural ecosystems to agriculture (Mannion 1998), as well as health and environmental concerns about pesticide use (Menzies and Bélanger 1996; Mari *et al.* 1996; Raupach and Kloepper 1998). The challenge to agriculture involves increasing productivity while minimizing expansion, improving production economics, and reducing negative environmental impacts (Dubos 1992; Mannion 1998).

To increase production without expansion requires further intensification of existing agricultural systems (Mannion 1998). Greenhouse crop production is highly intensive, with increased use of inputs allowing yields reaching nearly 40 times that of a similar area of field crop production (Paulitz and Bélanger 2001). Greenhouse production offers the opportunity to increase production without increasing the land base under production. Increased use of inputs in greenhouse crop production, particularly chemical inputs, still represents an environmental concern. Fungicides are commonly used in the management of fungal diseases of greenhouse crops and a reduction in fungicide use would reduce the negative environmental impact of greenhouse crop production. Reducing fungicide use may also reduce production costs, as pesticide applications can be expensive (Smith *et al.* 2002).

Reductions in fungicide use requires the development of alternatives to fungicides for greenhouse crop disease management and may also involve integrating disease management strategies. Integrated approaches to plant disease management practices are based on reducing fungicide use through cost effective control decisions using a variety of control options. Smith *et al.* (2002) defines integrated approaches as ecologically based management that combines biological, cultural, and chemical tools to minimize economic, health and environmental risks. An ecological, integrated approach to disease control is important to reduce fungicide use in greenhouse plant disease management.

Biological control is an important component of integrated strategies and an especially good fit for greenhouse crop production. Greenhouse production offers the possibility to modify the environment to influence the efficacy of biological control agents (Paulitz and Bélanger 2001; Punja 1997). Insect pest control in some greenhouses is almost completely accomplished through the use of biological control (Calpas *et al.* 2002; Menzies and Bélanger 1996). Effective biological control agents for greenhouse crop diseases, still need to be developed (Paulitz and Bélanger 2001).

Botrytis cinerea Pers., (teleomorph: *Botryotinia fuckeliana* (de Bary) Whetzel), is a cosmopolitan fungal plant pathogen with a very wide host range, able to parasitize and cause disease in over 200 hosts (Coley-Smith *et al.* 1980; Jarvis 1980a). *Botrytis cinerea* is capable of affecting all plant parts and attacking crops at almost any growth stage as well as in storage (Maude 1980). Diseases caused by *B. cinerea* are commonly known as grey mould or botrytis blight.

Botrytis cinerea is a common pathogen of greenhouse ornamental and vegetable crops including greenhouse tomato (*Lycopersicon esculentum* Mill.) (Hausbeck and Moorman 1996; Howard *et al.*1994). *Botrytis cinerea* infects tomato leaflets, petioles, stems, flowers and fruits (Howard *et al.*1994). Grey mould of greenhouse tomato can be devastating to a greenhouse operation. Wounds are particularly sensitive to infection by *B. cinerea* and the fungus can readily infect wounds on petioles caused by routine plant pruning and can progress into the stem (Howard *et al.*1994; Utkehde and Mathur 2002). Stems can be girdled by the developing lesions disrupting the flow of nutrients, often resulting in the death of the plant (Howard *et al.*1994; Utkehde and Mathur 2002).

In Canada, grey mould of tomato can be controlled by fungicide applications, however resistance of *B. cinerea* to fungicides is widespread (Elad *et al.* 1992). Management of the environment to prevent disease development, although theoretically possible, has not proved to be a successful approach in high yielding crops (Paulitz and Bélanger 2001). There are no cultivars resistant to *B. cinerea* (Elad and Evensen 1995). Crop rotation is not effective because of the wide host range of this fungus and because greenhouse operations are often specialized to one crop species. Alternative control methods for *B. cinerea* are needed and biological controls are desirable.

There are no biological controls currently registered for the management of *B. cinerea* in greenhouse crops in Canada. Research has been conducted on a number of biological control agents to determine their efficacy against *B. cinerea* in greenhouse tomato (Dik and Elad 1999; Elad *et al.* 1995; O'Neill *et al.* 1996; Utkhede *et al.* 2001; Utkhede and Mathur 2002). Much of this work included commercial biological control strains of *Trichoderma harzianum* Rifai, T-22 and T-39. These commercial strains of *T. harzianum* are marketed under the trade names RootShield® (T-22) (United States) and Trichodex® (T39) (Israel) (Harman and Björkman 1998). RootShield® has recently received registration in Canada for the suppression of root diseases caused by *Pythium* spp. Pringsh., *Fusarium* spp. Link ex Gray, and *Rhizoctonia solani* Kühn in greenhouse crops. Trichodex® is registered for the control of *B. cinerea* but does not have a Canadian registration.

Another product for the biological control of *B. cinerea* is Serenade®, based on *Bacillus subtilis* (Ehrenberg) Cohn strain QST713, and another organism that is being investigated for commercialization is *Ulocladium atrum* Preuss (Paulitz and Bélanger 2001).

Trichoderma spp. are common in soils worldwide, under differing environmental conditions, and living on various substrates (Hjeljord and Tronsmo 1998). This apparent high degree of ecological adaptability has been identified as one of the reasons why *Trichoderma* spp. are attractive candidates for a variety of biological control applications (Hjeljord and Tronsmo 1998). However, even after careful screening, *Trichoderma* spp. biocontrol candidates often show variable performance in field trials (Hjeljord and Tronsmo 1998). It has been suggested that the most appropriate criterion for selection of an effective biological control agent may be consideration of the specific environment in which the biological control agent would be expected to perform (Andrews 1990; Andrews 1992, Paulitz and Bélanger 2001).

This review discusses greenhouse tomato production, *B. cinerea* as a plant pathogen, response of the host plant to infection by *B. cinerea*, and *Trichoderma* spp. as biological control agents for *B. cinerea*. This discussion is presented as the background to the present study that developed an effective biological control agent for the management of *B. cinerea* in greenhouse tomato.

1.2 Tomato

Tomato (*Lycopersicon esculentum* Mill.) originated in South America and due to its value as a crop it has been distributed throughout the world (Taylor 1986). Selection and breeding to improve the adaptation of this tropical plant to different environments as well as improved quality and disease resistance has been ongoing for 200 years (Stevens and Rick, 1986). Domestication is believed to have started in Mexico (Taylor, 1986). The cherry tomato (*L. esculentum*. var. *cerasiforme* Dunal) originates in Central America and is the direct ancestor of modern cultivated tomato forms (Taylor 1986). Cherry tomatoes are the only wild tomatoes found outside of South America and domestication has clearly included considerable selection for larger fruit (Taylor 1986).

Tomato cultivars can be classed as determinate or indeterminate based on their growth habit. Determinate cultivars produce a branching system that has limited growth to a final form of a bush (Atherton and Harris 1986). Indeterminate cultivars produce branching systems that have the capacity to grow indefinitely that produces a prostrate growth habit (Atherton and Harris 1986). Greenhouse cultivars are indeterminate and require constant maintenance and physical support of the plants to allow for long term fruit production.

1.3 Greenhouse crop production

Greenhouse crop production includes a wide variety of ornamental and vegetable crops (Hausbeck and Moorman 1996; Maan 2001). Greenhouse production is based on high value crops (Menzies and Bélanger 1996; Paulitz and Bélanger 2001), and is a fast growing component of agriculture (Maan 2001; Papadopoulos and Dorais 2001).

Worldwide, there are over 300 000 ha under greenhouse production with 50 000 ha under sophisticated greenhouse production systems (Paulitz and Bélanger 2001). Greenhouse vegetable production in Canada is based on sophisticated greenhouse production systems. The Canadian greenhouse vegetable industry was valued at $600M in 2000 (Papadopoulos and Dorais 2001). Alberta is Canada's fourth largest greenhouse vegetable producing area with over $20M in sales in 2001 (Maan 2001; Papadopoulos and Dorais 2001). Tomatoes are Canada's primary greenhouse vegetable with over 468 ha (Papadopoulos and Dorais 2001), including 8.8 ha under production in Alberta (Maan 2001).

Sophisticated greenhouse operations make use of technology to intensify production, allowing control of the environment and precise delivery of water and nutrients (Calpas 2002). This control allows for very dense crop plantings and to maximized yield through ongoing optimization of the environment (Calpas 2002). Greenhouse tomato crop planting densities are in the range of 2.5 to 2.7 plants m^{-2}, resulting in yearly yield totals of over 60 kg m^{-2} of tomatoes by experienced growers (Calpas 2003).

Providing growing conditions that are optimal for plant growth poses a challenge because these environmental conditions can also be optimal for disease development (Menzies and Bélanger 1996; Paulitz and Bélanger 2001). It can be very difficult for growers to maintain an optimal environment for crop production without also favouring disease development (Menzies and Bélanger 1996; Paulitz and Bélanger 2001).

The situation is further complicated when greenhouse ventilation is reduced in order to minimize heating costs. Energy costs are a significant concern to greenhouse growers in Canada (Maan 2001; Paulitz and Bélanger 2001). When greenhouse ventilation is reduced, the high planting density results in a sustained increase in the relative humidity providing ideal conditions for *B. cinerea*. The routine pruning and harvesting of the crop opens wounds that can be colonized by *B. cinerea* and if unchecked, stem cankers can develop resulting in plant death. Every dead plant represents approximately 24 kg of lost tomato yield. In a 2000 survey of Alberta greenhouse growers, 47% of growers identified *Botrytis* sp. as their primary disease concern (Mann 2001).

When epidemics of *B. cinerea* occur, other methods of disease management are required. Chemical control options for greenhouse growers are limited because the greenhouse market is a relatively small market for pesticide manufacturers (Paulitz and Bélanger 2001). The fungicides registered for control of *B. cinerea* in greenhouse tomato in Canada are Rovral® (iprodione, Bayer), Ferbam® (ferric dimethyidithiocarbamate, UCB Chemicals) and a Benlate ® (benomyl, Du Pont)–Manzate® (mancozeb, Du Pont) mix. The Benlate®-Manzate® combination is not registered for use on hydroponic greenhouse tomato crops. Hydroponic crop production systems include any system where the application of water is integrated with the delivery of fertilizer, regardless of the substrate the plant is growing in (Calpas 2002). More management options are required, especially when considering the capacity of *B. cinerea* to develop resistance to fungicides.

Development and registration of chemical or biological controls is an involved process representing a significant investment for companies (Harman 2000; Sexsmith 1998). Investing in the registration of biological controls can be attractive because of increasing environmental and health concerns. Greenhouse growers are very sensitive to pesticide use issues particularly regarding worker safety and government mandated reentry times that can disrupt work schedules (Harman 2000). Biological controls with no known human health risks and no reentry period requirements can have an advantage over chemical controls if they are cost effective and efficacious (Harman 2000).

1.4 Biological management of plant disease

Biological management of plant disease has been broadly defined as the reduction of inoculum or disease-producing activity of a pathogen accomplished by or through one or more organisms other than man (Baker and Cook 1974). Andrews (1990) offered a narrower definition, "the application of one or more microorganisms to control plant pathogens". The latter definition more accurately describes the current state of research and application of biological control of plant pathogens. The use of microorganisms is the basis of biological control of plant disease (Elad and Chet 1995).

The idea of using microorganisms as biological control agents of plant pathogens has been recognized as arising from research in 1932 discovering that *Trichoderma lignorum* would parasitize a number of soil-borne fungi in culture (Adams 1990). It was suggested that control of certain plant pathogens could be accomplished by augmenting soil with this mycoparasite (Adams 1990). Since this time *Trichoderma* spp. have figured prominently in efforts to develop biological controls as a tool for the management of a variety of plant pathogens (Dubos 1992; Hjeljord and Tronsmo 1998).

1.4.1 Mechanisms of biological control

Bacteria, fungi, and yeasts have been studied as potential biological control agents (Punja 1997; Whipps and Lumsden 1991). Microorganisms as biological control agents are believed to function against plant pathogenic organisms with three main antagonistic mechanisms of action. These mechanisms are: competition, antibiosis and mycoparastism (Harman 2000; Whipps and Lumsden 1991). This list has recently been expanded to include induction of plant resistance mechanisms (Dubos 1992; Harman 2000; Hjeljord and Tronsmo 1998; Punja 1997). Harman (2000) included inactivation of the pathogen's enzymes as a separate biological control mechanism but this could arguably be included under antibiosis.

Adjunct or indirect mechanisms of activity are also discussed in the context of biological control agents and include improvements in water and nutritional status through a more efficient root system (Jacobsen and Backman 1993; Harman 2000; Howell 2003). Harman (2000) also included solubilization and sequestration of organic nutrients by the biological control agent as a mechanism of biological control.

Competition occurs when two or more microorganisms demand more of the same resource than is available (Hjeljord and Tronsmo 1998). Competition can be difficult to demonstrate as a mechanism of action and is usually inferred when there is antagonism in absence of direct mycoparasitism or antibiosis (Harman 2000; Howell 2003). Competition is considered important for the control of *B. cinerea* (Dubos 1992).

As a facultative saprophyte, *B. cinerea* capably develops on senescing leaves and flowers, and uses these materials as a nutrient base from which to invade the plant (Dubos 1992). *Trichoderma* spp. are also capable colonizers of dying plant tissue and has been shown to prevent saprophytic colonization of *B. cinerea* (Dubos 1992). Competition for nutrients and space has long been considered as a mechanism of biological control activity by *Trichoderma* spp. (Harman 2000). Competition for nutrients at wound sites on plants would also likely be considered important for the control of *B. cinerea*.

Antibiosis can include the production of toxins and lytic enzymes that reduce pathogen growth or survival (Punja 1997). The boundary of what is included as a different mechanism of antagonism is not sharp. The activity of enzymes is included under mycoparasitism by some even if it involves the killing of target hyphae a short distance from the antagonist (Harman 2000). Strains of *Trichoderma* spp. produce secondary compounds that inhibit other microorganisms at a distance (Hjeljord and Tronsmo 1998). Certain strains of epiphytic bacteria introduced on grapes are thought to protect the grapes by the production of compounds that inhibit the germination of *B. cinerea* (Dubos 1992).

Mycoparastism is the direct penetration of plant pathogen propagules by the antagonist (Punja 1997). It is considered an important mode of action by *Trichoderma* spp. and enzymes certainly play an important role (Hjeljord and Tronsmo 1998; Harman

2000). Mycoparasitism has not been widely described for biological control activity against *B. cinerea* with the exception of *Trichoderma* spp. (Dubos 1998).

The induction of host resistance in cucumber (*Cucumis sativus* L.) against *Pythium aphanidermatum* Edson, has been demonstrated by applying weakly virulent isolates of *Pseudomonas corrugata* Roberts and Scarlett (Zhou and Paulitz 1994). *Trichoderma harzianum* has also been shown to induce general defence responses in cucumber plants (Yedidia *et al.* 1999).

Increased tolerance to plant stress has been postulated for the activity of a highly rhizosphere competent strain of *T. harzianum* T-22 marketed as RootShield®. The plants under disease pressure from *Phytophthora* spp. were able to grow better in the presence of *T. harzianum* than without it (Harman 2000). This strain of *T. harzianum* is also known to enhance nitrogen use efficiency in corn and can also solubilize Zn^{2+}, Mn^{4+}, Fe^{3+} and Cu^{2+} making these elements more readily available to plants (Harman 2000). Plants having access to these nutrients may be better able to resist disease in a similar manner as improved resistance through a more efficient root system.

There are a number of known and proposed mechanisms by which antagonistic microorganisms function against plant pathogens as biological control agents. Other mechanisms will likely be added as research increases the understanding about the complex interactions of antagonists and pathogens in specific systems of application.

1.4.2 Selecting a biological control agent.

Expectations of the performance of biological control agents are partially based on our assessments of their mechanisms of action (Harman 2002). If assumptions are made that specific agents have a limited number of mechanisms then it could be concluded that the activity is specific to particular pathogens or crops (Harman 2000). If a broad range of mechanisms exist, there may be reason to conclude that the biological control agent could have broader application (Harman 2000).

It is generally believed that success of a biological control agent in the field requires that the agent not only be successful at controlling the pathogen, but also be adapted to the environment (Andrews 1992; Elad and Chet 1995; Harman 2000;

Jacobsen and Backman 1993; Whipps and Lumsden 1991). *Trichoderma* spp. are frequently numerous and even dominant in agricultural soils because they likely have numerous mechanisms to exploit their ecological niche (Harman 2000). An understanding of mechanisms responsible for biological control in a given system is essential to interpret the basis for success and could be the foundation to improve performance (Andrews 1990; Elad and Chet 1995; Harman 2000; Jacobsen and Backman 1993; Whipps and Lumsden 1991).

There is almost no understanding of the mechanisms of biological control that operate in the field (Andrews 1990). Much of the understanding of the mechanisms of biological control has come from *in vitro* studies (Elad and Chet 1995). However, it has been demonstrated that the mechanism of biological control that is exhibited by a biological control agent *in vitro* may not be the same mechanism that is exhibited by the same agent *in vivo* (Edwards and Seddon 2001). *In vitro* studies are often used in the search and selection of biological control agents (Elad and Chet 1995; Paulitz and Bélanger 2001). However *in vitro* performance is not a reliable predictor of *in vivo* field performance of the biological control agent (Andrews 1990; Elad and Chet 1995).

Reliable field performance of potential biological control organisms has been one of the limiting factors in the development of biological control products (Elad and Chet 1995; Jacobsen and Backman 1993). Improved field performance will result from an increased understanding of the ecology of the biological control organisms in the community in which it is expected to perform (Andrews 1990). The challenge is to understand the interactions that govern distribution and abundance of the biological control agent and the pathogen (Andrews 1990). This is a complex area of study, however this work is necessary to identify predictors that will allow for selection of biological control agents with a more reasonable expectation of success in the field (Andrews 1990).

The phyllosphere is an especially challenging environment for biological control of plant disease (Andrews 1990). Commercial products for biological control agents are few, and especially few for foliar plant diseases (Elad and Chet 1995, Punja 1997). Andrews (1990) discussed the potential for success of biological control in the phyllosphere and stated that it "may not be a false hope, but neither is it confirmed as a realistic goal". Commercial success may prove to be the ultimate determiner of the success of biological control in the phyllosphere (Andrews 1990, Paulitz and Bélanger 2001).

In the absence of basic ecological knowledge to predict successful biological control in the phyllosphere, empirically testing mass numbers of organisms to determine what works, is the other option (Andrews1990). The methods to test and select potential biological control agents are critical considerations for success (Andrews 1990; Andrews 1992, Elad and Chet 1995; Hjeljord and Tronsmo 1998). Andrews (1990) stated that the objectives of biological control candidate selection is to "get the best match at the outset between the organism and the environment where it will have to function".

1.5 *Botrytis cinerea*

Botrytis cinerea is a competent plant pathogen and saprophyte and is found wherever its host crops are grown (Coley-Smith 1980; Jarvis 1977). The geographic distribution of the genus ranges from cool temperate regions to subtropical (Jarvis 1977). Most records are associated with cool-temperate and warm-temperate zones and this is thought to be due to the distribution of the economic host plants that are grown throughout these regions (Jarvis 1977).

Botrytis spp. are not generally regarded as soil fungi (Jarvis 1977). However *Botrytis cinerea* commonly occurs in the soil as a small proportion of the fungal population (Domsch and Gams 1972; Jarvis 1977). Reports of *B. cinerea* isolation from soil are mainly associated with the upper layers (Jarvis 1977). *Botrytis cinerea* has also been recovered from rhizosphere soil (Parkinson and Thomas 1965) and from bean (*Phaseolus vulgaris* L.), barley (*Hordeum vulgare* L.) and cabbage (*Brassica oleracea* L.) roots examined at 5 and 10 days after germination (Parkinson *et al.*1963). It is interesting to note that *B. cinerea* was also present as an occasional seed surface contaminant by Parkinson and Thomas (1965). *Botrytis cinerea* is considered to sporulate poorly in the soil and mycelia surviving less than a week in soil (Jarvis 1977).

The presence of *B. cinerea* in soil is primarily associated with its host plant and decaying plant residues (Jarvis 1977). *B. cinerea* is a foliar pathogen and its saprophytic activity on plant residue is primarily above the soil (Jarvis 1977).

1.5.1 Taxonomy

Botrytis is an old genus, erected by Micheli in 1729 and is one of the first described genera of fungi (Jarvis 1977; Jarvis 1980a). Persoon validated the genus in 1801 and included one of Micheli's species, *B. cinerea* (Jarvis 1977; Jarvis 1980a). *B. cinerea* is a conidial (asexual) fungus and is also a pleomorphic fungus, one that exists in two independent forms (anamorphs), with *Botryotinia fuckeliana* (de Bary) Whetzel being the teleomorph (sexual stage) (Jarvis 1977; Jarvis 1980a). With pleomorphic fungi, both stages are treated as separate taxonomic and nomenclatural entities identified by separate binomials (Jarvis 1977; Jarvis 1980a).

The full citation of *B. cinerea* presented by Hennebert (1973) including synonyms and the perfect state is:

> *Botrytis cinerea* Pers., Syn. meth. Fung. 690. 1801 = *Haplaria grisea* Link *in* Mag. Ges. naturf. Freunde, Berlin 3: 11. 1809 = *Polyactis vulgaris* Link, *loc. cit.* 16. 1809 = *Phymatotrichum gemellum* Bon., Handb. Mykol. 116. 1851. = *Botrytis fuckeliana* Buchw. *in* K. Vet.- og Landbohøjsk. Aarsskr. 1949: 147. 1949; conidial state of *Botryotinia fuckeliana* (De Bary) Whetzel *in* Mycologia 37: 679. 1945.

Ainsworth *et al.* (1971) in "Dictionary of Fungi" cites *B. cinerea* Persoon ex Fries. Hennebert does not include Fries in his citation. Fries often worked with written descriptions of fungi and did not save type specimens of fungi he validated (Bisby 1945). Not including a type specimen with a description in support of a binomial is no longer acceptable (Agerer *et al.* 2000; Greuter *et al.* 1994).

Hennebert's (1973) revision of the genus describes how the genus *Botrytis*, lectotypified by *B. cinerea* Persoon, which first included only five species, ballooned to accommodate over 380 species. Hennebert (1973) describes the priority of Persoon's binomial for the fungus and identifies the incorrect synonymous names for *B. cinerea.* One reason for all the apparent confusion about *B. cinerea* was the lack of a universally accepted type (Jarvis 1980a). Hennebert (1973) attributes the lectotype species of the genus, *B. cinerea* to Clements and Shear in 1931, although Jarvis (1980a) notes that Groves and Loveland in 1953 apparently missed the lectotype of Clements and Shear and designated another specimen in the Persoon herbarium as the lectotype.

Hennebert (1973) also explains that some of the confusion surrounding *B. cinerea* is that the fungus "presents many different aspects" from the early stage of conidiophore development to full maturity. Some of the genera (*Phymatotrichum* and *Polyactis*) synonymized with the genus *Botrytis* were based on immature stages of *B. cinerea* (Hennebert 1973).

The magnitude of the work involved in the revision of *B. cinerea* is conveyed in Hennebert's (1973) paper, as is the frustration with workers who are not diligent in their use of binomials. Hennebert (1973) describes how the genus *Phymatotrichum* was correctly synonymized within the genus *Botrytis*, which should have effectively taken the genus *Phymatotrichum* out of circulation. Hennebert (1973) also states that after all this work was completed, the genus *Phymatotrichum* was resurrected by Bloss in 1970, "who seems to accept any assigned species". The use of correct names and avoiding errors continues to be a source of frustration for mycologists (Agerer *et al.* 2000).

As *B. fuckeliana* is undoubtedly the sexual stage in the life cycle of *B. cinerea* (Hennebert 1973; Jarvis 1977; Jarvis 1980a; Faretra and Grindle 1992), most taxonomists recommend that the scientific name of the sexual stage should be preferred to that of the asexual stage (Faretra and Grindle 1992). *Botryotinia fuckeliana* should replace *B. cinerea* as the Latin binomial for the fungus causing grey mould (Faretra and Grindle 1992). However, *B. cinerea* is a familiar binomial, recognized around the world by many mycologists and plant pathologists (Faretra and Grindle 1992).

There are precedents for retaining the name of the asexual stage of a fungus (Faretra and Grindle 1992) however both *B. cinerea* and *B. fuckeliana* should be included in publications on *B. cinerea* (Faretra and Grindle 1992).

1.5.2 *Botrytis cinerea* and *Botryotinia fuckeliana*

When compatible strains of *B. cinerea* are crossed, apothecia of *B. fuckeliana* develop from typical *B. cinerea* sclerotia. Single ascospores produced by the apothecia give rise to typical colonies of *B. cinerea* and single ascospore cultures are self-sterile (Groves and Loveland 1953).

Sclerotia are hard, black, and firmly attached to the substrate. They are variable in size and shape, rounded to elongated, 1-15 mm in length, sometimes forming continuous masses (Groves and Loveland 1953). Apothecia can develop singly or in clusters and are 3-15 mm tall and 1.5-7.0 mm in diameter (Groves and Loveland 1953). They are dark brown in colour with a paler margin.

Typical *B. cinerea* conidiophores can also arise from sclerotia as well as mycelium. Conidiophores are erect, brown in colour and usually branched near the tips. The tips of conidiophores are swollen and rounded, giving rise to grayish-brown conidia in botryose clusters. Conidia are one celled, ellipsoidal to ovoid, 8-18 x 5-9 µm (Groves and Loveland 1953).

1.5.3 *Botrytis cinerea* pathogenesis

All *Botrytis* spp. are important plant pathogens but only *B. cinerea* is a broad spectrum plant pathogen, hosts including almost all of the dicotyledons, many of the monocotolydons and some of the Pteridophyta (Jarvis 1977; Jarvis 1980b; Verhoeff 1992). Most *Botrytis* spp. have a more restricted host range with some species being host species specific (Jarvis 1977; Jarvis 1980b;Verhoeff 1992). *B. cinerea* has attracted more attention than any other species within the genus, attacking field crops, greenhouse crops, flowers and fruit. *Botrytis cinerea* causes crop losses both during production and storage (Verhoeff 1992).

Botrytis cinerea is a necrotrophic plant pathogen having a critical requirement for dead tissue or exogenous nutrients supplied in an aqueous environment such as thin films of water on wound sites (Elad 1997; Heale 1992; Jarvis 1977; Jarvis 1980b).

The infection process of *B. cinerea* has been considered as a process of three consecutive stages (Kamoen 1992). The first stage is the germination of conidia on the plant surface, followed by penetration of the host epidermis and expanding colonization of the host tissue (Kamoen 1992).

High relative humidity and the presence of free water on plant surfaces are probably the single most important environmental requirements governing the germination of conidia of *B. cinerea* (Jarvis 1980b; Blakeman 1980). The presence of free water is essential for leaf infection of a number of different hosts by species of *Botrytis* spp. (Jarvis 1980b; Blakeman 1980). The optimum temperature for germination and growth of *B. cinerea* is between 20 and 25°C (Blakeman 1980). Optimum temperatures for infection are between 10 and 20°C, with infections occurring at 2 to 25°C (Elad *et al.* 1996).

Germination of conidia is stimulated by the presence of nutrients such as provided by pollen and senescing tissues as well as sugars and amino acids associated with wounds (Blakeman 1980; Epton and Richmond 1980; Jarvis 1977; Kamoen 1992). The effect of predisposing environmental conditions on the plant can also influence availability of nutrients to stimulate germination of conidia and infection. Low light levels in greenhouse tomato were shown to predispose leaves to infection by *B. cinerea* by acting as a stressor that caused increased nutrient leakage from the leaf (Shafia *et al.* 2001).

Inhibition of germination can occur when conidia are present in high numbers (Blakeman 1980; Kamoen 1992). More than 10^5 spores mL^{-1} leads to self-inhibition of germination, however high spore concentrations can be more effective in causing infections (Kamoen 1992). The release of nutrients, as a result of germination and penetration of some of the conidia acting to stimulate the subsequent germination of conidia, has been suggested as the mechanism of overcoming the self-inhibition of germination at high spore concentrations (Kamoen 1992).

The ability of *B. cinerea* conidia to germinate decreases with increasing age. Infectivity of older conidia is also reduced (Blakeman 1980; Kamoen 1992). The reduction in germination has been attributed to a loss of endogenous nutrients (Blakeman 1980; Kamoen 1992). Conidia from 40-day-old cultures of *B. cinerea* were not able to germinate as readily as conidia from 10-day-old cultures (Jarvis 1980b, Blakeman 1980). In addition, conidia from 40-day-old cultures of *B. fabae* were not as infective as conidia from 10-day-old cultures (Jarvis 1980b, Blakeman 1980). Both the reduced germination and infectivity of older conidia can be restored through the addition of exogenous nutrients (Blakeman 1980; Kamoen 1992).

Swelling is the first visible sign of germination followed by germ tube development (Epton and Richmond 1980). After 10 hours of incubation in a glucose and mineral salt medium, conidia had increased in diameter by 21% and most had developed a germ tube (Epton and Richmond 1980). Germ tubes emerged from random points on the conidium of *B. cinerea* (Epton and Richmond 1980).

Penetration of intact host cuticle can occur directly via the germ tube or through the formation of an appressorium (Jarvis 1977). A mucilaginous gel often surrounds both the germ tube and appressorium (Jarvis 1977; Kamoen 1992), and is probably composed of ß (1-3) or ß (1-6) glucans (Kamoen 1992). Degradation of the cuticle under germ tubes occurs and both mechanical and enzymatic action are involved (Jarvis 1977; Kamoen 1992). Cutinases and pectinases present in the germination fluid of the conidia and young germ tubes appear to be important for penetration (Kamoen 1992).

Germ tubes of *B. cinerea* are also able to penetrate host tissue through stomata, lenticels and all types of wounds (Jarvis 1977). *Botrytis cinerea* is especially capable of colonizing wounds and any mechanical damage to plant tissues will predispose them to infection (Jarvis 1977; Kamoen 1992). Wounds provide an aqueous environment, a ready supply of exogenous nutrients and direct access to internal plant tissues (Jarvis 1977; Kamoen 1992).

Once established, *B. cinerea* can expand rapidly in the host tissue. As the hyphae penetrate the tissues, the host cells are killed in advance of the colonization or after the hyphae have grown beyond them (Kamoen 1992). *Botrytis cinerea* produces an array of enzymes and toxins that are involved in expansion and colonization of host tissues. Enzymes are primarily responsible for the maceration of plant tissue and toxins are considered the main factors responsible for killing cells in advance of the hyphae (Jarvis 1977).

An array of enzymes are involved in pathogenesis of *Botrytis* spp. Enzymes that degrade the cell wall and middle lamella include pectin methyl esterases, endo- and exo-polygalacturonases (endo- and exo- PGs), and pectic lyases (Kamoen 1992; Verhoeff 1980). Laccases degrade, solubilize, and depolymerize lignin as well as detoxify some other host chemical defences such as phytoalexins (Pezet *et al.* 1991; Viterbo *et al.* 1992). A few hemicellulases are known to be produced (Verhoeff 1980). *Botrytis cinerea* is apparently able to break down cellulose, however the cellulases involved and their specific activities are also not well understood (Kamoen 1992; Verhoeff 1980). Breakdown of cellulose is a complex reaction involving several enzymes (Kamoen 1992; Verhoeff 1980). After the cellulose is solubilized into glucan chains ß(1-3), glucanase and cellobiase are involved in the further breakdown to glucose or cellobiose (Kamoen 1992; Verhoeff 1980). Acid proteases act on cell wall proteins (Verhoeff 1980) and are important in causing the death of the plant cell (Movahedi and Heale 1990).

The ability to recognize and respond to cues from a changing environment is a basic requirement of pathogenesis and survival (Gronover *et al.* 2001; Zheng *et al.* 2000). The activity of cell-wall degrading enzymes is not always found in association with infection by *Botrytis* spp. (Verhoeff 1980), the requirements for pathogenesis apparently can vary with host. However, requirements for broad-spectrum pathogenesis by *B. cinerea* exist. The ability to secrete extracellular protease and endo-pectic lyase enzymes appears to be critical for successful pathogenesis in a number of host species (Gronover *et al.* 2001; Movahedi and Heale 1990).

The enzyme compliment of different strains of *B. cinerea* can vary. Between 1 and 13 polygalacturonase isozymes were secreted from a number of different *B. cinerea* strains, with growth medium as the major factor behind the expression of different isozymes (Van der Cruyssen and Kumoen 1992). It is unclear just how many endo- and exo-PGs are produced by *B. cinerea* as the numbers vary both with strains and age of cultures (Rha *et al.* 2001). Some of the PGs are expressed constitutively and others are induced by the presence of galacturonic acid and conditions of low pH (4.5-5.5) (Rha *et al.* 2001, Wubben et al 2000).

The presence or absence of enzymes is regulated by the activity of genes, and multiple genes can be involved in the production and regulation of a family of enzymes. At least 6 genes for endo-PGs have been cloned and the existence of multiple exo-PG genes is also probable (Rha *et al.* 2001). The involvement of these genes in the pathogenicity and aggressiveness of *B. cinerea* is not known (Rha *et al.* 2001).

The BCG1 and BCG2 genes are involved in controlling different signal transduction pathways in *B. cinerea*, for the secretion of extracellular proteases and rate of colonization, respectively (Gronover *et al.* 2001). Neither gene was identified as an absolute requirement for pathogenesis, however when either gene was absent, *B. cinerea* aggressiveness was reduced (Gronover *et al.* 2001). In contrast, single genes that are critical for pathogenicity in *B. cinerea* have been identified. The BMP1 gene is thought to control the morphological requirements for infection by *B. cinerea* such as germ tube and appressoria development (Zheng *et al.* 2000). Conidia of BMP1 deletion mutants are apparently normal and germ tube development occurs, but the mutants are unable to infect, even through wounds (Zheng *et al.* 2000). In addition BMP1 may also be involved in regulating the expression of cell wall degrading enzymes (Zheng *et al.* 2000).

As a prerequisite to successful colonization in some hosts, there is evidence that *B. cinerea* is able to detoxify plant phytoanticipans, preformed fungitoxic compounds important to plant defence (Quidde *et al.* 1999). A number of these phytoanticipans are saponins, and glycosylated steroidal or triterpenoid compounds that can breakdown fungal cell membranes, and can exclude pathogens unable to degrade the saponins (Agrios 1997). *Botrytis cinerea* is able to deglycosylate the tomato phytoanticipin, a-tomatine as well as saponins from potato and oats (Quidde *et al.* 1999). The reduced virulence on tomato of a strain of *B. cinerea* lacking tomatinase activity was used to postulate that this enzyme might form the basis of host specificity among strains (Quidde *et al.* 1999).

The production of toxins by *B. cinerea* and their role in pathogenesis is not well understood. Citric acid, oxalic acid, thiourea, and urea are the substances primarily discussed as the toxins produced by *B. cinerea* (Jarvis 1977; Verhoeff 1980; Kamoen

1992). The activity of these substances has been associated with plant cell death in advance of hyphae (Jarvis 1977; Verhoeff 1980). Inoculation of leaves with culture filtrates containing these substances produced similar symptoms as seen with initial inoculation with *B. cinerea* (Jarvis 1977; Verhoeff 1980).

More recently, it has been shown that the toxins associated with *B. cinerea* infection on a range of host plants can also be associated as an indirect result of the activity of enzymatic degradation of the host cells. The activity of a endo-pectic lyase and aspartic proteinase on the host cell walls caused the release of host cell wall components that were toxic to host cells (Movahedi and Heale 1990).

It has also been demonstrated that the presence of *B. cinerea* induces high levels of hydrogen peroxide and generation of hydroxyl radicals that form the basis for an non-enzymatic oxidative attack of host cells during the early stages of infection (von Tiedemann 1997). It was not clear whether *B. cinerea* produced the hydrogen peroxide directly or whether it caused enhanced production by the plant (von Tiedemann 1997). The toxic activity of the oxyradicals occurs in advance of the detection of cell-wall degrading enzymes and is considered to be the toxic factor most likely responsible for cell death (von Tiedemann 1997). The effect of oxyradicals is oxidative tissue damage. This damage is non-specific and the ability of *B. cinerea* to induce oxyradical formation was suggested as being a contributing factor to the wide host range of *B. cinerea* (von Tiedemann 1997).

Certain enzyme compliments and specific toxins may be a requirement for successful pathogenesis on specific hosts and may form the basis for host range specificity in some *B. cinerea* strains. Some genes are an absolute requirement for pathogenesis while others are not. As the genes involved, and requirements for the pathogenesis of *B. cinerea* are identified and their actions studied, more will be understood about the host pathogen interaction in *B. cinerea*.

Strains of *B. cinerea* were shown to vary in virulence against grape although isolates collected from tomato were able to cause disease on grape (Derckel *et al.* 1999). Other work using random amplified polymorphic DNA (RAPD) analysis to characterize *B. cinerea* isolates collected from grapes, apple, blueberry, and tomato suggest some host specialization in *B. cinerea* (Thompson and Latorre 1999). There may be degrees of host specificity in wild type strains but it is clear that some broad based mechanisms of enzymatic and toxic activity exist within *B. cinerea*. The wide host range of this pathogen may require a specific set of mechanisms for pathogenesis of specific hosts, mechanisms that are broadly applicable to many hosts, or both.

1.5.4 Grey mould of greenhouse tomato

1.5.4.1 Symptoms and signs

Tomato leaflets, petioles, stems, flowers and fruits can become infected by *B. cinerea* (Howard *et al.* 1994). Older tissues are more susceptible to attack than younger ones (Howard *et al.* 1994). Infections on leaflets can expand to include the entire leaf, and can continue down the petiole into the main stem. Wounds are particularly open to infection, as they serve as a ready entry points for the pathogen.

Leaf lesions begin as brown coloured circular spots, as the lesions expand they take on a more water-soaked appearance especially under conditions of high humidity (Howard *et al.* 1994). Condiophores and conida develop on the entire surface of the lesion causing the distinctive grey mould signs (Howard *et al.* 1994). Infected tissues eventually collapse, wither, and fall to the floor or lodge in the plant.

Infected petiole and stem wounds become water-soaked, and dark brown to black in appearance. Lesions on petioles and stems can progress rapidly, expanding a few millimeters to several centimeters in length (Howard *et al.* 1994). In commercial tomato greenhouses in Alberta, lesions developing on petioles wounded by routine leaf pruning can develop up to 3 centimeters in 1 week under conditions of high humidity (Calpas unpublished). Under dry conditions, stem lesions take on a light brown to grey appearance and can repeatedly develop conidiophores and conidia under subsequent cycles of high humidity (Calpas unpublished). The progression of petiole lesions into the stem is particularly damaging when the lesion girdles the stem causing death of the plant.

The most common symptom on green fruit is a ghost spot, and this symptom can also occur on ripe fruit (Howard *et al.*1994; Jones *et al.* 1991). Ghost spots are characterized by a small necrotic fleck surrounded by a light colored "ring" or "halo" a few millimetres in diameter (Howard *et al.* 1994). The halo is conspicuous against the background of the green or orange-red colour of the tomato fruit. Ghost spot occurs when *B. cinerea* penetrates the fruit and creates a visible lesion, but further spread is prevented, even during ripening (Elad and Evensen 1995).

1.5.4.2 Disease cycle

Conidia, ascospores, mycelium and sclerotia are all important for dispersal of *B. cinerea* although only conidia seem to be involved in dispersal in the greenhouse (Kerssies and Bosker-van Zessen 1997; Hausbeck and Moorman 1996). In greenhouses in Israel, sclerotia are rarely found on plants infected with *B. cinerea* and are not produced during the summer (Elad *et al.* 1992). Conidia can originate from sources inside and outside the greenhouse (Kerssies and Bosker-van Zessen 1997). *Botrytis cinerea* can survive between successive crops on infected plant residue and act as a source of primary inoculum (Elad *et al.* 1992). *Botrytis cinerea* can also be introduced into the new crop on tomato transplants purchased from commercial propagation greenhouses.

Dispersal of conidia can occur through mechanical action associated with routine crop maintenance (Hausbeck and Moorman 1996; Kerssies 1992). In the absence of grower activity, peak concentrations of conidia in the air occur between mid-morning and mid-afternoon, corresponding to the period associated with a rapid decrease in relative humidity (Hausbeck and Moorman 1996). Conidia are also dispersed through air currents, water droplets, and insects (Kerssies 1992).

Conidia contacting the plant surface are able to germinate and invade almost immediately, but can remain dormant for up to 3 weeks before germinating (Hausbeck and Moorman 1996). Ungerminated conidia or quiescent infections can become active when environmental conditions favour disease development in the crop or post-harvest (Hausbeck and Moorman 1996; Keller *et al.* 2003). Conidia produced from lesions within the crop serve as the main source for secondary infections although conidia originating from outside the greenhouse can also be responsible (Hausbeck and Moorman 1996; Kerssies *et al.* 1997).

1.5.4.3 Disease management

Management of grey mould in greenhouse crops involves manipulation of the environment to prevent disease development. The presence of free water is required for germination of the conidia, and conditions of high humidity and free water favour development of the fungus and infection (Blakeman 1980, Epton and Richmond 1980). Environmental control to maintain the relative humidity below 80% and prevent the formation of free water on the plants will reduce the incidence of grey mould (Howard *et al.* 1994). This can be accomplished through heating and venting the greenhouse in an ongoing process of dehumidification, however this practice is costly. To be effective the environmental control must be uniform throughout the greenhouse. The formation of microclimates that favour disease development within the tomato crop canopy can still occur.

Diseased plant material or infested plant residue should be removed from the greenhouse as these materials can serve as sources of inoculum.

When epidemics of *B. cinerea* occur, other methods of disease management are required. Chemical control options for greenhouse growers are limited because the greenhouse market is a relatively small market for pesticide manufacturers (Paulitz and Bélanger 2001). The fungicides registered for control of *B. cinerea* in greenhouse tomato in Canada are Rovral® (iprodione, Bayer), Ferbam® (ferric dimethyidithiocarbamate, UCB Chemicals) and a Benlate ® (benomyl, Du Pont)–Manzate® (mancozeb, Du Pont) mix. The Benlate®-Manzate® combination is not registered for use on hydroponic greenhouse tomato crops. Hydroponic crop production systems include any system where the application of water is integrated with the delivery of fertilizer, regardless of the substrate the plant is growing in (Calpas 2002).

More management options are required, especially when considering the capacity of *B. cinerea* to develop resistance to fungicides. Development of fungicide resistant strains of *B. cinerea* in greenhouse tomatoes is a constant concern (Utkhede and Mathur 2002).

Development and registration of chemical or biological controls is an involved process representing a significant investment for companies (Harman 2000; Sexsmith

1998). Investing in the registration of biological controls can be attractive because of increasing environmental and health concerns. Greenhouse growers are very sensitive to pesticide use issues particularly regarding worker safety and government mandated reentry times that can disrupt work schedules (Harman 2000). Biological controls with no known human health risks and no reentry period requirements can have an advantage over chemical controls if they are cost effective and efficacious (Harman 2000).

1.6 Plant defence responses

Plants have developed diverse and complex responses to attack by plant pathogens. Plant resistance is a dynamic phenomenon depending on many biochemical components (Wurms *et al.* 1999). Defence responses include both pre-existing and induced responses that can be localized at the infection site or systemic. Agrios (1997) presents a comprehensive review of these general defence responses of plants to pathogens. Plant responses to reduce infection by *B. cinerea* involve components from all the categories of general responses and are reviewed in detail by Elad (1997).

Successful pathogenesis by *B. cinerea* depends on overcoming the host plant defences and is characterized by spreading, necrotic lesions (Thomma *et al.* 1999). Plant species and cultivars can differ in their response to *B. cinerea* and many mechanisms involving families of genes are involved in a resistant response (Dixon and Lamb 1990). Increased resistance can be associated with enhanced activity of individual components. In relatively resistant hosts, the action of a single phytoalexin or a single chitinase expressed in transgenic plants was responsible for increased resistance to *B. cinerea* (Stevenson and Haware 1999; Kishimoto *et al.* 2002). Although genetic variation for resistance to *B. cinerea* is observed between plant species no gene-for-gene resistance has been identified (Elad and Evensen 1995).

In some cases resistance may depend on improved recognition and or appropriate signaling of the presence of *B. cinerea* infection (Dixon and Lamb 1990). There can also be different plant responses to different strains of *B. cinerea*, with defence responses delayed in the presence of more virulent *B. cinerea* (Derckel *et al.* 1999). Increased virulence in strains of *B. cinerea* can be caused directly by relatively more aggressive attack mechanisms of the pathogen or can result from a failure of the host to recognize the more virulent strain.

The ability to escape detection or avoid plant defence responses by minimizing damage to the host has been suggested as a mechanism of pathogenesis in biotrophic plant pathogens (Osbourn 1996). Pathogenesis by necrotrophs such as *B. cinerea* involves the death of host tissue and continued expansion of the necrotic lesion in advance of the pathogen. A necrotroph is more likely to run a gauntlet of host defence responses in the process of successful pathogenesis.

Rapid recognition and response is an important component of plant defence, however pathogens may exploit advantages in the process of the response. Ethylene is a plant growth regulator that is involved in the induction of plant defence responses (Ecker and Davis 1987; Thomma *et al.* 1999). Ethylene production often increases in response to infection by plant pathogens (Ecker and Davis 1987; Thomma *et al.* 1999).

Systemic acquired resistance (SAR) responses induced by pathogens are dependent on signaling pathways and the defence responses are different depending on the signaling pathway that is activated (Zimmerli 2001). Salicylic acid is involved in signaling SAR responses as are jasmonic acid and ethylene-dependent signaling pathways.

Ethylene mediated plant defence responses were important components of a resistant response to *B. cinerea* in Arabidopsis plants (Thomma *et al.* 1999; Zimmerli *et al.* 2001). Arabidopsis mutants defective in the jasmonic acid, ethylene-dependent SAR mediated transduction pathways were more susceptible to infection by *B. cinerea* (Thomma *et al.* 1999; Zimmerli *et al.* 2001). Although plants with functioning ethylene transduction pathways were more resistant to *B. cinerea*, they were still susceptible to infection (Thomma *et al.* 1999; Zimmerli *et al.* 2001).

Conversely, ethylene is also known to increase incidence of *B. cinerea*. Host plant ethylene production has been demonstrated to increase disease development in a number of plants including tomato (Elad 1997). Ethylene accelerated plant senescence is associated with increased membrane permeability and could increase tissue susceptibility to infection by *B. cinerea* (Elad 1997). Reduction of ethylene levels in the storage atmosphere reduced levels of disease development by *B. cinerea* on flowers (Elad 1997).

The generation of active oxygen species is reported to be one of the earliest plant defence responses to infection (Malolepsza and Urbanek 2002). Active oxygen species are considered to play an important role in the hypersensitive reaction (HR) where localized cell death limits the spread of pathogens (Govrin and Levine 2000). They are also involved in other plant defence responses including wound healing and the formation of physical barriers to infection (Lurie *et al.* 1997). Lignification and suberization occurs through the polymerization of phenols that is catalyzsed by a cell wall associated peroxidase (Lurie *et al.* 1997).

The HR defence response is considered to be an important factor limiting the growth of biotrophic pathogens as these pathogens require compounds from living cells (Govrin and Levine 2000). Pathogens that do not trigger the HR response are able to cause disease in the plant (Govrin and Levine 2000). It was postulated that the HR response is effective against biotrophs but it may aid the colonization by necrotrophs (Govrin and Levine 2000). It has been demonstrated that *B. cinerea* is able to take advantage of the plant HR response for rapid colonization of *Arabidopsis* (Govrin and Levine 2000). This view of active oxygen species aiding colonization by *B. cinerea* agrees with the observations of von Tiedemann (1997), however, he speculated that the oxygen species may have been produced by *B. cinerea*.

The effect of active oxygen species on *B. cinerea* in tomato has demonstrated that active oxygen species are an important part of a resistant response reducing the rate of colonization (Malolepsza and Urbanek 2000; Malolepsza and Urbanek 2002). Plant cells are protected against oxidative damage by a complex antioxidant system including the enzymatic oxidants superoxide dismutase, peroxidases and catalase (Malolepsza and Urbanek 2002). Heat inactivation of peroxidase in heat-treated tomato fruit caused increased susceptibility to *B. cinerea* (Lurie *et al.* 1997). Active oxygen species may serve to protect the plant against invasion by necrotrophs like *B. cinerea* only when the plants antioxidant system is functioning optimally.

Botrytis cinerea must also have anti oxidant defences as there is evidence for the role of active oxygen species in plant defence as well as the pathogenesis of *B. cinerea.* It is not likely that an indiscriminant offensive tool would be a component of *B. cinerea* pathogenesis unless the fungus also had the capability to protect itself from active oxygen species. The actual role of active oxygen species may be determined somewhat arbitrarily. If the fungus is successful the active oxygen species would be seen as a component of successful pathogenesis. If the plant is successful in resisting infection, the active oxygen species is therefore seen as a component of plant defence. The reality probably rests in the balance and is determined by the generation and presence of active oxygen species and the antioxidant resources of the combatants.

The role of components of the plant defence response, including ethylene and active oxygen species, in the host-pathogen interaction is complex and clearly not one-sided. Understanding the physiology of plant resistance to *B. cinerea* should allow for the development of more resistant cultivars. Enhancing crop plant resistance through breeding for desirable traits is an important component of disease control (Agrios 1997).

Different resistance mechanisms are involved at different developmental stages of the plant and this makes breeding for resistance to *B. cinerea* difficult (Elad and Evensen 1995). Considering the diversity of plant chemical and physical responses to infection by plant pathogens including *B. cinerea*, it is not likely that manipulating individual factors will allow for complete resistance (Elad and Evensen 1995). The plant defence response relies on a number of processes and strategies to tip the balance in favour of the plant to prevent disease. Strategies to control disease caused by *B. cinerea* will also require a variety of strategies to tip the balance in favour of host plant resistance (Elad and Evensen 1995).

1.7 *Trichoderma* spp.

Trichoderma spp. are extremely common, rapid growing, soil fungi found in agricultural, prairie, forest, salt marsh, and desert habitats throughout the world (Klein and Eveleigh 1998). They are generally saprophytes, but can attack other fungi (Klein and Eveleigh 1998). They have minimal nutritional requirements and produce a large number of secondary metabolites (Klein and Eveleigh 1998; Sivasithamparam and Ghisalberta 1998). The genus is generally reported to be resistant to a number of pesticides including fumigants such as methyl bromide and fungicides including captan (Klein and Eveleigh 1998). In addition *Trichoderma* spp. are widely known to degrade insecticides including DDT, and Malathion (Klein and Eveleigh 1998).

Strains of *Trichoderma* spp. can degrade hydrocarbons (Klein and Eveleigh 1998). *Trichoderma* spp. can degrade a variety of complex plant and fungal compounds including cellulose, pectin, starch, xylan, chitin and glucans (Benítez *et al.* 1998; Klein and Eveleigh 1998). The quantity of the enzymes secreted by *Trichoderma* spp. has been described as being massive (Klein and Eveleigh 1998). *Trichoderma* spp. enzymes are employed in industrial processes. Industrial use of cellulases from strains of *T. reesei* began in 1980 with current widespread application in the production of textiles, foods and animal feed (Mäntyla *et al.* 1998; Galante *et al.* 1998a; Galante *et al.* 1998b).

Commercial application of *Trichoderma* spp. also extends into crop protection as biological control agents with a number of commercial products (Howell 2003). *Trichoderma harzianum* is one of the most common species of the genus and is well known as for its biological control capabilities against fungal plant pathogens (Gams and Meyer 1998; Howell 2003). *Trichoderma harzianum* has also been shown to have biological control potential against plant pathogenic nematodes (Sharon *et al.* 2001) as well as bark beetle vectors of the Dutch elm disease pathogen (Jassim *et al.* 1990).

1.7.1 *Trichoderma harzianum*

1.7.1.1 Taxonomy

T. harzianum is a conidial or asexual fungus (Gams and Bisset 1998). The genus *Trichoderma* as was introduced by Persoon in 1794 and the association of the genus with teleomorphs in the *Hypocrea* was recognized in 1865 (Gams and Bissett 1998). In addition to *Hypocrea*, *Trichoderma* spp. teleomorphs are known in the related genera *Podostroma* and *Sarawakus* although the teleomorph of *T. harzianum* is not known (Gams and Bissett 1998).

Rifai (1969) made the first serious effort to morphologically distinguish speices, or more correctly "species aggregates" as he was aware that the taxa he studied were not biological entities but groups of closely related organisms (Gams and Bissett 1998; Rifai 1969). One of the species aggregates Rifai (1969) identified was *T. harzianum*. *Trichoderma harzianum* was neotypified by Gams and Meyer (1998) using an isolate from the type locality, the holotype, referred to by Rifai (1969) having been lost. There is considerable variability in strains included under *T. harzianum* and the application of "species" to *T. harzianum* still applies in the broad sense (Gams and Bisset 1998; Gams and Meyer 1998). The most recent taxonomic key of the genus still includes the species aggregate concept for *T. harzianum* (Gams and Bissett 1998). Molecular techniques are showing promise in resolving species within the genus *Trichoderma*.

The citation for *T. harzianum* presented by Gams and Bissett (1998) is:

Trichoderma harzianum Rifai, Mycol. Pap. 116:38. 1969.

1.7.1.2 *Trichoderma harzianum* as a biological control agent.

The biological control potential of *Trichoderma* spp. was first recognized in the 1930s in a study involving *T. lignorum* (Howell 2003). In addition to *T. harzianum*, a number of *Trichoderma* spp. have been investigated for biological control activity. These include *T. virens* (Howell *et al.* 1999; Wilhite *et al.* 2001)(formerly *Gliocladium virens* (Gams and Bissett 1998)), *T. atroviride* (Giuliano *et al.* 2001), *T. viride* (Shanmugam *et al.* 2001; Yates *et al.* 1999), *T. hamatum* (Gracia-Garza *et al.* 1997), and *T. longibrachiatum* (Migheli *et al.*1998).

Trichoderma spp. are primarily soil fungi and the biological control potential of these fungi has been evaluated against a number of soil-borne pathogens. These include *Sclerotium rolfsii* Sacc. (Elad *et al.*1980; Inbar and Chet 1995), *Rhizoctonia solani* Kuhn (dal Soglio *et al.* 1998; Elad *et al.* 1980), *Macrophomina phaseolina* (Tassi) Goid. (Khan and Gupta 1998) and *Pythium* spp. (Naseby *et al.* 2000; Thrane *et al.* 2000) and many others. A number of commercial products for the control of root pathogens based on *T. harzianum* are marketed throughout the world (Paulitz and Bélanger 2001) The one that is most commonly reported in the scientific literature is RootShield® based on *T. harzianum* strain T-22. RootShield® is reported to be effective in controlling a number of root pathogens including, *Pythium* spp., *Rhizoctonia solani* and *Fusarium* spp., on a number of crops (Harman 2000; Paulitz and Bélanger 2001).

The RootShield® strain T-22 of *T. harzianum*, has also been demonstrated to be effective in reducing the severity of fusarium wilt of tomato caused by *Fusarium oxysporium* f. sp. *lycopersici* W.C. Snyder & H.N. Hans. (Larken and Fravel 1998).

Trichoderma harzianum has also been used as a biological control agent against foliar plant pathogens, in particular, those caused by *B. cinerea* (Paulitz and Bélanger 2001). It is interesting that so much work on biological control of *B. cinerea* in a variety of crops has been conducted using a microorganism that is not a usual component of the phylloplane microflora (Dubos 1992). Considerable work on the biological control of *B. cinerea* has been conducted on greenhouse vegetable crops, particularly tomato, and much of this work has been conducted with a commercial strain of *T. harzianum*, strain T-39, marketed as Trichodex®.

Trichoderma harzianum strain T-39, initially isolated from a cucumber fruit (O'Neill *et al.* 1996), was developed in Israel primarily as a biological control agent of *B. cinerea* in grapes (Paulitz and Bélanger 2001). Trichodex® is registered in a number of countries for the control of *B. cinerea* (Elad and Chet 1995). Trichodex® has been reported to give good control of *B. cinerea* in greenhouse crops including cucumber (Dik and Elad 1999) and tomato (Dik and Elad 1999; O'Neill *et al.* 1996). Most of the work evaluating Trichodex® has been conducted in unheated greenhouses in areas where high humidity and the formation of free water on the plants is common (Dik and Elad 1999).

Other research conducted to evaluate Trichodex® in heated tomato and cucumber greenhouses in the Netherlands has reported control at least as good as fungicides (Dik and Elad 1999). However, recent studies evaluating Trichodex® to control *B. cinerea* greenhouse tomatoes in Spain have reported performance not significantly different from the untreated control (Moyano *et al.* 2003). The overall performance of Trichodex® from at least 60 greenhouse trials has been assessed as providing control of *B. cinerea* and frequently at levels obtained by fungicides (Harman and Björkman 1998).

Strains of *Trichoderma* spp. are generally considered to be only effective against specific pathogens and that this can be due to the specific environmental conditions and to the pathogen-specific activity of *Trichoderma*'s enzymes and antibiotics (Hjeljord and Tronsmo 1998). However, Harman (2000) reported that single strains of *Trichoderma* spp. are capable of controlling a wide range of pathogens and that one strain is all that may be required for all situations. Harman (2000) further speculates that costs of commercialization are such that one strain could be all that a company can afford to bring to market.

It is worth noting that strain T-22 was developed in Harman's laboratory and that Harman co-founded BioWorks, Inc., to develop and market commercial biological products (Harman 2000). A number of products based on the single strain of *T. harzianum* have been developed for the greenhouse, row crop, and turf industries (Harman 2000). RootShield® is the best known of these products but another product, TopShield®, has recently been developed for use against *B. cinerea* (Harman 2000). Strain T-22, was developed by a protoplast fusion of a mutant isolate of *T. harzianum* strain recovered from Columbia and a *T. harzianum* strain recovered from soil in New York (Paulitz and Bélanger 2001; Harman 2000). Retail sales of T-22 products in 1999 were reported at $3 million with an expectation to increase substantially over the coming years (Harman 2000).

Studies using T-22 found that control of *B. cinerea* was about as good as the standard fungicide iprodione, but T-22 in combination with a surfactant performed better than either iprodione or T-22 alone (Harman 1998). Recent work with *T. harzianum*, strain T-22 for the biological control of *B. cinerea* has been conducted in greenhouse tomatoes in British Columbia, Canada (Utkhede *et al.* 2001; Utkhede and Mathur 2002). Both Canadian studies demonstrated that strain T-22 has the potential to control *Botrytis* stem canker in greenhouse tomato.

A review of the scientific literature regarding the performance of commercial products based on *T. harzianum*, particularly RootShield® and Trichodex® illustrate that biological control products can be successfully commercialized. It is also clear that these commercial single strains of *T. harzianum* can be efficacious against a variety of pathogens including both root and foliar pathogens. Inconsistent control has been obtained against some pathogens in the same crops in greenhouse environments. Inconsistent control of *B. cinerea* in greenhouse tomato and cucumber has been reported for *T. harzianum* strain T-39 (Trichodex ®). This indicates that other biological control products, based on *T. harzianum* or other organisms could be better suited for certain crop-environment combinations. It is not likely that one or two biological control products will provide the required level of control in a wide variety of environments. In the big picture, there will probably always be room in the market for another effective biological control agent.

1.7.1.3 Mechanisms of biological control by *Trichoderma* spp.

Mechanisms involved in the biological control activity of *Trichoderma* spp. include mycoparasitism, antibiosis, competition, induction of host defences and indirect effects of improving the plant health status. These mechanisms are reviewed in detail by Harman (2000) and Howell (2003).

Trichoderma spp. produce a host of secondary compounds, many of which have antibiotic activity (Sivasithamparam and Ghisalberti 1998). Many different enzymes are also produced by the genus (Chet *et al.* 1998). It has been demonstrated that there is synergistic activity between enzymes and toxins in the antagonistic activity of *Trichoderma* spp. against pathogens, including *B. cinerea* (Howell 2003).

The mechanisms of antagonisms can vary with the strain of *Trichoderma* spp. and not all the mechanisms necessary for optimum biological control are found in the same strain (Howell 2003). The importance of any given mechanism is dependent on the strain, the target organism and the environment (Harman 2000; Hjeljord and Tronsmo 1998; Howell 2003). This has to be taken into account when a biological control agent is assessed. The assessment should include the intended target organism in the intended environment (Hjeljord and Tronsmo 1998). The mechanisms involved in successful biological control are not readily assayed in the laboratory (Howell 2003).

Based on this information, one has to question if it is reasonable to expect that single strains of *Trichoderma* spp. will provide adequate control in a variety of pathogens on a variety of hosts, in a variety of environments.

1.7.1.4 Effect of *Trichoderma* spp. on plants

Certainly the most obvious direct effect of biological control strains of *Trichoderma* spp. is the antagonism of a plant pathogen's activity to cause disease on the plant. The effects of inducing plant resistance and increasing plant vigour through better access to nutrients or reduction in nutrient stress are generally discussed as mechanisms of the biological control activity of *Trichoderma* spp., direct or indirect. However, it is important to recognize that *Trichoderma* spp. can have negative effects on plants. *Trichoderma harzianum* secondary compounds have been shown to inhibit growth of wheat seedlings, and to be toxic to beans, corn, and tobacco plants (Bailey and Lumsden 1998).

Trichoderma spp. are rarely considered to be plant pathogens (Bailey and Lumsden 1998). A strain of *T. viride* was identified as a minor pathogen to germinating seedlings of cucumber, pepper and tomato (Menzies 1993).

1.7.1.5 *Trichoderma* spp. as pathogen of commercial mushrooms

Green mould is a serious disease of common mushroom *Agaricus bisporus* Lange caused by *T. harzianum* (Boyle 1999; Ospina-Giraldo *et al.* 1999). However not all strains of *T. harzianum* are implicated in causing the disease, the biological control strains are not considered aggressive to *A. bisporus* (Ospina-Giraldo *et al.* 1999).

1.8 Objectives

The overall goal of this thesis research was to develop a biological control for *B. cinerea* on greenhouse tomato using a native Alberta strain of *Trichoderma* sp. To achieve this goal the objectives were as follows:

1. Collect strains of *B. cinerea* and develop a method to assess virulence against tomato and other plant species. Use the RAPD-PCR technique to evaluate the genetic diversity of the *B. cinerea* strains, and investigate the relationships revealed by the RAPD analysis. Examine whether the RAPD-PCR differentiation of the *B. cinerea* isolates corresponds to virulence or host specificity.

2. Collect and identify a number of *Trichoderma* spp. strains. Develop a method to assess biological control potential against *B. cinerea* in greenhouse tomato crops. Determine the level of diversity of the *Trichoderma* spp. strains with respect to biological control potential. Use RAPD-PCR to evaluate genetic diversity and relationships between the *Trichoderma* spp. strains with particular interest in assessing biological control potential.

3. Develop a model system to grow greenhouse tomatoes according to commercial standards to obtain commercial levels of production for use as the environment to develop an effective biological control for *B. cinerea*.

4. Using the greenhouse tomato model system, develop a *Trichoderma* spp. biological control for *B. cinerea* using the criterion that the biological control agent must perform as well as fungicides.

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Chapter 2

Characterization of pathogenicity and virulence of *Botrytis cinerea* strains

2.1 Introduction

Botrytis cinerea, (teleomorph *Botryotinia fuckeliana*) is widely distributed in temperate regions of the world (Jarvis 1980a). This plant pathogen displays a high degree of genetic (Alfonso *et al.* 2000; Kerssies and Bosker-van Zessen 1997; Muñoz *et al.* 2002) and phenotypic diversity (Gronover *et al.* 2001; Movahedi and Heale 1990; Rha *et al.* 2001), and is able to parasitize over 200 host plants. *Botrytis cinerea* causes grey mould on a variety of greenhouse crops (Hausbeck and Moorman 1996, Howard *et al.*1994).

Alberta has a diverse greenhouse industry including ornamental and vegetable crops with over 380 operations involving 109 ha under greenhouse production (Maan 2001). Grey mould diseases caused by *B. cinerea* affect all above ground plant tissues and *B. cinerea* is the main foliar pathogen of concern for Alberta greenhouse growers (Maan 2001). Botrytis stem cankers can cause the death of infected plants if the cankers develop to girdle the stem. *Botrytis cinerea* stem canker can be devastating to commercial greenhouse tomato crops (Utkhede *et al.* 2001). Stem canker caused by *B. cinerea* can also be devastating to commercial geranium growing greenhouses (Hausbeck and Pennypacker 1991).

Disease assessment is an important component of plant pathology involving accurate measurement of plant disease, and is useful in quantifying the differences between treatments to select disease management practices (Mayee and Dater 1989). Disease assessment scales are often used for measuring disease severity, with qualitative scales and quantitative scales representing the 2 general approaches used. Qualitative scales use a subjective division to separate disease severity into categories, and quantitative scales are based on a measurement of a quantitative trait (O'Brien and van Bruggen 1992).

Quantitative scales based on the length of lesion development have been used to assess *B. cinerea* stem canker development in tomato stem pieces as well as on whole plants (O'Neill *et al.* 1996; O'Neill *et al.* 1997). Direct measurements of *B. cinerea* stem canker development on tomato stems have also been used as an assessment of disease development (Utkhede *et al.* 2001). Measurement of *B. cinerea* lesion development has also been used to assess disease development in other hosts including geranium (*Pelargonium* × *hortorum* L.H. Bailey) (Hausbeck and Pennypacker 1991; Uchneat *et al.*1999) and blueberry (*Vaccinium ashei* Reade) (Smith 1998).

Studies evaluating potential biological control agents against *B. cinerea* require strains of *B. cinerea* for use as the target organism(s). In studies where *B. cinerea* is applied in the assays, single strains of *B. cinerea* have often been used (Dik and Elad 1999; Elad *et al.* 1995; O'Neill *et al.* 1996; Shafia *et al.* 2001; Utkhede *et al.* 2001; Utkhede and Mathur 2002). Other assays relied on natural populations of *B. cinerea* for infection (Köhl *et al.* 1998; Moyano *et al.* 2003). In all assays it is clear that pathogenic strains of *B. cinerea* were used, or were present. None of the studies report selecting a target strain based on characterization of virulence on the host of interest. There are no reports of studies involving a large number of *B. cinerea* strains recovered from a variety of host plants.

A prerequisite to the development of a successful biological control agent for *B. cinerea* in greenhouse tomato is the collection of a virulent strain of the pathogen for use as the target strain. Hence the objectives of this study were;

1. Collect strains of *B. cinerea* and evaluate diversity in their pathogenicity and virulence on greenhouse tomato and other plant species.

2. To identify a virulent strain of *B. cinerea* for use as the target strain in the development of a biological control for *B. cinerea* on greenhouse tomato.

2.2 Materials and methods

2.2.1 Strains of *Botrytis cinerea*

Botrytis cinerea strains were collected from diseased plant material obtained from a number of greenhouse and field sources throughout Alberta (Table 2-1). Five strains were collected from each source. The strains were recovered from the diseased plant tissue and established in pure culture using single spores. Cultures were maintained on 20 mL of potato dextrose agar (PDA) (BBL™) in 8.5 cm diameter Petri plates at 20 °C under approximately 9 hr of natural light per day. Cultures were exposed to 14 hr of UV light (Philips 40 W lamp, F40T12/BLB) for the first 3 days of culture to induce sporulation. *Botrytis cinerea* covered the plate with mycelial growth in 1 week and mature conidia covered the entire plate at 3 weeks.

All strains were identified as *Botrytis* spp. using Hennebert's (1973) description of the gross colony morphology of the type species for genus, *B. tulipae* Lind. The strains were identified as *B. cinerea* using the microscopic description of conidiophores and conidia from the lectotype proposed by Groves and Loveland (1953).

2.2.2 Characterization of colony morphology of *Botrytis cinerea* strains

Observations of differences in colony characteristics of the strains of *B. cinerea* were made as a means of comparing the different strains. Strains where subjectively characterized based on the production of relatively darker or lighter mycelium, for sclerotial production as well as in conidial production.

Differential production of conidia was assessed according to Hansen (1938) for use as a convenient method to group *B. cinerea* strains. Strains were characterized into 3 culture types, namely C, M or C/M. "C" culture types were characterized by relatively heavy conidiation, "M" culture types were primarily mycelial and represented the other end of the spectrum producing relatively less conidia. "C/M" culture types represented the intermediate strains. All subjective assessments were made when the cultures were 1 week old. The cultures used in this assessment were not exposed to UV light.

2.2.3 Characterization of pathogenicity and virulence of *Botrytis cinerea* strains using stem piece bioassays

2.2.3.1 Plant material

Tomato plants (cv. Quest, De Ruiter Seeds) were seeded on November 15 and grown and maintained in a 120 m^2 computer controlled (Argus Control Systems) greenhouse under commercial conditions according to Calpas (2003). Cucumber (*Cucumis sativus* L.) cv. "Flamingo", petunia (*Petunia* × *hybrida* L.) cv. "Purple Wave" and geranium (*Pelargonium* × *hortorum* L.H. Bailey) cv. "Kim" plants were grown in a commercial peat-based growing medium (Pro Mix 'BX'®, Premier Horticulture). The cucumber plants were started from seed, the petunia plants were started from 2 cm square plugs and the geraniums were started from rooted cuttings. The plants were grown in 25 cm diameter pots with 1 plant per pot. The pots were placed on benches in a greenhouse under natural light at 20 to 24 °C during the day and 19 to 21°C during the night. The plants were watered with a nutrient solution as required. The nutrient solution was made using 20-20-20 fertilizer (Plant-Prod®) at a rate of 1 g/L, delivering 200 ppm of nitrogen, 88 ppm of actual phosphorus and 166 ppm of actual potassium. No insecticides or fungicides were applied to the plants.

All cultivars selected for use in this study were cultivars that were grown by commercial growers.

2.2.3.2 Stem piece bioassays

A tomato stem piece bioassay similar to that described by O'Neill *et al.* (1996) was used to characterize the virulence of the *B. cinerea* strains. Length of stem colonized by *B. cinerea* was used to characterize the virulence as more virulent strains were expected to aggressively colonize the stem piece compared to less virulent strains (O'Neill *et al.* 1996). Lateral stems, approximately 50 cm long were collected from the tomato plants for use in the stem piece bioassay. The tomato stem bioassay was conducted twice.

A subset of 35 strains was selected for further evaluation against cucumber (Table 2-5), geranium (Table 2-6) and petunia (Table 2-7). The strains were selected to include tomato strains that were identified as highly virulent and less virulent using the stem piece bioassay as well as a number of strains initially recovered from cucumber, petunia and geranium.

Stem piece bioassays using the other host plant species (cucumber, petunia, geranium) were conducted in a manner similar as for tomato. Experiments for each additional host plant were conducted separately and were conducted once.

Three-centimeter-long sections of stem were placed upright in sterile, flat-bottomed Pyrex™ culture tubes (Corning™ #9850-25). Five mm diameter agar plugs were removed from a 3-week old sporulating culture of each *B. cinerea* strain and were placed on the top of the stem pieces. The agar plugs were positioned on the top of the cut stem piece with the *B. cinerea* spores placed in direct contact with the freshly cut surface, and a cap placed to close each vial. Four replicate treatments of each *B. cinerea* strain were evaluated in the trial. The vials were arranged in a randomized design on a bench top.

The stems were kept at 20°C under 12 hours of light (natural light supplemented by cool-white fluorescent light) per day for 1 week at which time the length of stems colonized by the *B. cinerea* strains were measured. Stem pieces were considered as being infected when lesions greater than 2 mm in length were evident on the cut stem ends as reported by O'Neill *et al.* (1996).

2.3 Statistical analyses

Data were analyzed by an analysis of variance (ANOVA) using the general linear model (GLM) procedure (SAS Institute Inc). The model considered lesion length as a function of trial date, strain of *B. cinerea*, the interaction of date and strain and experimental error. Trial date, strain of *B. cinerea* and the interaction were treated as random factors.

The model used for the analysis of the data from the stem piece bioassays conducted on cucumber, geranium and petunia, considered lesion length as a function of the strain of *B. cinerea*. Strain was treated as a random factor in the analyses. All analyses were conducted using a 5% level of significance.

2.4 Results

2.4.1 Collection and identification of strains of *Botrytis cinerea*

One hundred and sixty strains of *B. cinerea* were obtained from 32 collection locations throughout Alberta (Fig. 2-1). The strains were recovered from 20 host species growing in the greenhouse (23 locations) and field (9 locations) (Table. 2-1). Strains collected from greenhouse locations were collected from diseased plants inside the greenhouse. Strains collected from field locations were collected from diseased plants from the same area of the field. Thirty-eight strains of *B. cinerea* were generally collected from greenhouse tomato.

The culture development of every putative strain of *B. cinerea* was generally consistent with Hennebert's (1973) colony description of the type species for the genus:

> "*Colonies* effuse, at first white to grayish, then dark brown; hyaline to brown, septate."

The mycelial (M) strains did not develop the dark brown colony color as distinctly as all other strains. The development of the dark brown color is associated with the development of further production of conidiophores and condia. M strains produced relatively few conidia and remained lighter in color, however where conidiophore and conidia production did occur the color progression was consistent with Hennebert's description.

Microscopic examination of all strains was also consistent with Groves and Loveland's (1953) microscopic description of their proposed lectotype of *B. cinerea*:

> "Conidiophores arising from the mycelium or from the sclerotia, erect, brown, septate, simple or usually branched toward the tip, the tip rounded or slightly swollen, 5-22μ in diameter, 1-3 mm. In height; conidia grayish-brown in mass, nearly

> hyaline under the microscope, ellipsoid to ovoid, one-celled, borne on small stalks arising from the tips of the conidophores and forming botryose clusters, 8-14-(18) x (5)-6-9μ."

On the basis of microscopic studies and culture morphology all 160 strains were determined to be *B. cinerea*. Micrographs illustrating botryose conidial clusters are presented in Figures 2-2 and 2-3.

2.4.2 Characterization of colony morphology of the *Botrytis cinerea* strains

The results of the subjective culture assessments are presented in Table 2.2. The strains displayed variability in the expression of culture characteristics. Most of the strains had lighter coloured mycelium in culture with exception of the AL strains, PA strains, and strains CA3 and EO2. All categories of conidial production were represented by the strains with most of the strains classed as intermediate in the C/M category. There was also good representation from strains that were sclerotial producers and those that were not. Generally those strains that were considered strong producers of conidia (C strains) were less likely to produce sclerotia and M strains were more likely to produce sclerotia. Intermediate C/M strains exhibited variability in production of sclerotia. For example, the strains from the TM location all produce sclerotia while the strains from the VW location did not and both sets of strains were collected from greenhouse tomato.

Strains from the same location generally tended to be similar in expression of culture characteristics (Table 2-2) although there are exceptions as all the EO location strains varied in the mycelium colour and production of sclerotia although all EO location strains were categorized as C/M.

There were no apparent patterns of association between the differential culture characteristics.

2.4.3 Characterization of pathogenicity and virulence of the *Botrytis cinerea* strains

Photographs of 1 week-old cultures, representing the 3 categories of sporulation (C, M, and C/M represented by strains LF1, LF5 and TX1 respectively), are presented in Figure 2.4. Table 2-3 contains morphological descriptions from the representative strains from each of the 3 categories.

The 160 strains of *B. cinerea* exhibited a range of virulence against tomato as demonstrated by the tomato stem piece bioassay (Table 2-4, Fig. 2-4). The data was approximately normally distributed.

Of the 38 strains of *B. cinerea* collected from tomato, 9 (LF5, LF1, LF4, BN3, BT2, BA4, BA1, BT1, BT3) were in the top 10 most virulent strains against tomato. The most virulent strain was the tomato strain LF5 and 3 strains from the LF location, placed in the top 10. The BT strains, collected from greenhouse tomato, were also quite virulent with 3 of the 5 strains in the top 10. One of the strains (BN3) collected from greenhouse tomato from the BN location was in the top 10. However, the other BN strains were not as virulent and placed lower in the ranking. Tomato strains of *B. cinerea* included strains that ranged from highly virulent to non-pathogenic based on the tomato stem bioassay.

The ANOVA of the tomato stem piece bioassay (Table 2-4) demonstrates that all factors, date, strain and the date by strain interaction had a significant effect on lesion length. The partitioning of the variation in the data was 41% due to date, 2.9% due to strain, 24% to the date by strain interaction and 32% due to error.

These results indicated that with only 2.9% of the variation in the data due to strain, that the performance of the *B. cinerea* strains is more greatly affected by the trial date and date by strain interaction which account for 65% of the variation collectively. This underscores that the relationship between *B. cinerea* and its host is a complex interaction.

Botrytis cinerea strain SB4 from strawberry also placed in the top 10 most virulent strains. Strains obtained from different hosts demonstrated a distribution similar to that seen from tomato ranging from highly virulent to weakly virulent.

The cucumber stem piece bioassay also revealed a range of differentiation in virulence ranging from highly virulent to non-pathogenic (lesions less than 2 mm long). The most virulent strain on cucumber was the tomato strain LF4, with the 4 other LF strains, including LF5 in the top 10. Strains non-pathogenic on cucumber also included the tomato strains VW4 and TM1. Only 1 cucumber strain EO3 ranked in the top 10.

The ANOVA of the cucumber stem piece bioassay (Table 2-5) demonstrate the significant effect of *B. cinerea* strain on lesion length. Strain accounted for 46.2% of the variation in the data with 53.8% of the variation due to error.

Similarly, the geranium (Table 2-6) and petunia (Table 2-7) stem piece bioassay demonstrated that tomato strains highly virulent on tomato, including LF5, were also highly virulent on geranium and petunia. Strains recovered from geranium and petunia were pathogenic on geranium and petunia although they often displayed degrees of virulence.

The ANOVAs of the geranium and petunia stem piece bioassays also demonstrated the significant effect of *B. cinerea* strain. Strain accounted for 46.2% and 14.4% of the variation in the geranium and petunia bioassays respectively, with experimental error accounting for the balance.

There were no apparent associations between the culture characteristics and virulence and pathogenicity. Strains LF5 and LF4 were both grouped in the C/M category, had light coloured mycelium and produced sclerotia. Both strains were virulent on tomato, cucumber and petunia, but LF5 was also virulent on geranium where LF4 was weakly virulent. Strain BT1 was grouped as C/M had light coloured mycelium and was virulent on tomato, petunia and geranium and moderately virulent on cucumber. Strains EO1 and EO2 were both classed as C/M, both were moderately virulent on tomato but strain EO1 has light coloured mycelium and produced sclerotia where strain EO2 has dark mycelium and did not produce sclerotia. Examinations of all the strains display this variability of association among and with the culture characteristics and virulence.

2.5 Discussion

2.5.1 Colony morphology of the *Botrytis cinerea* strains

The strains of *B. cinerea* exhibited variation in the culture characteristics of mycelium colour, production of sclerotia and conidial production as assessed in this study. Culture characteristics of *B. cinerea* are known to be highly variable and characters can change substantially with changing culture conditions (Jarvis 1977; Jarvis 1980b). Conidia size, form and colony characteristics of *B. cinerea* can be changed by altering temperature and culture medium, and these morphological changes are reversible (Jarvis 1977). Hennebert (1973) stated that *B. cinerea* presents considerable variation in form during its development and these stages have been taken as representing distinct genera. Differences in culture characters of *B. cinerea* has lead to taxonomic confusion as later work has demonstrated the problems with using culture characteristics as taxonomic criteria (Jarvis 1977).

It is recognized that *B. cinerea* in culture can be grouped based on morphological characteristics including growth that is primarily mycelial, conidial or sclerotial (Jarvis 1977; Jarvis 1980b; Paul 1929). Hansen's (1938) description of categories differentiated *B. cinerea* based on relative degrees of sporulation. Paul (1929) examined mycelial, conidial and sclerotial strains of *B. cinerea* with respect to host specificity and did not find differences in virulence associated with the differential growth in culture. Based on this experience with *B. cinerea*, any determinations or associations based on or including characters of colony morphology are doubtful. However, the differences in colony characteristics demonstrated in this study serves to illustrate the natural diversity in *B. cinerea*.

2.5.2 Diversity in pathogenicity and virulence of the *Botrytis cinerea* strains

The stem piece bioassays provided a means to differentiate the strains of *B. cinerea* based on pathogenicity and virulence. The bioassays demonstrated that the virulence of some strains were high on all hosts while virulence of other strains varied across the range of the 4 host species. There was no clear evidence of host specificity although strains recovered from a plant species were generally pathogenic on that plant species in the bioassays. Some strains that were highly virulent on tomato were also highly virulent on some of the other hosts (strains LF5, LF4, SB4) and the converse was also true (strains TM1, TX2). These observations agree with the generally accepted view that *B. cinerea* is not host specific, expressing a high degree of variability in virulence and able to parasitize a wide range of hosts.

2.5.3 Basis for variation in *Botrytis cinerea*

Mutation is the primary cause of heritable variation in fungi and the 2 mechanisms that govern this variation in *Botryis* spp. and its perfect state *Botryotinia* spp. are sexual recombination and heterokaryosis (Lorbeer 1980). Heterokaryosis is the condition resulting from the anastomosis of cells of fungal hyphae from 2 strains that results in the exchange of nuclei so that hyphal cells contain 2 or more nuclei that are genetically different (Agrios 1997). Vegetative compatibility between strains is defined as the ability to form a viable heterokaryon by hyphal fusion (Fournier *et al.* 2003). The formation of a heterokaryon is considered an important first step in the parasexual cycle. Two genetically distinct nuclei fuse to form a diploid followed subsequent reversion to a haploid state that is accompanied by significant genetic exchange and new forms of the fungus (Leslie 1993).

Vegetative compatibility groups have been used to characterize population diversity in fungi (Di Primo *et al.* 2001; Fournier *et al.* 2003) including *B. cinerea* (Fournier *et al.* 2003). Heterokaryosis in *B. cinerea* has been demonstrated in culture plates (Fournier *et al.* 2003; Lorbeer 1980) and is recognized as a major source of variation in *B. cinerea* (Hansen 1938, Bergmans and Van Kan 1992; Hilber *et al.* 1994). Although heterokaryosis is widely accepted as a major source of variation in *B. cinerea* its role is not well understood (Delcán and Melgarejo 2002).

Vegetative compatibility and heterkaroyosis have also been studied in *Fusarium oxysporium* f.sp. *radicis-lycopersici* Jarvis and Shoemaker. A diversity of vegetative compatibility groups is present in this plant pathogenic fungus (Di Primo *et al.* 2001). Heterokaryons of *F. oxysporium* f.sp. *radicis-lycopersici* forming under culture plate conditions were demonstrated to be unstable leading to speculation that the diversity of vegetative compatibility groups present in *F. oxysporium* f.sp. *radicis-lycopersici* is not likely due to heterokaryosis (Di Primo *et al.* 2001). Heterokaryosis in *F. oxysporium* f.sp. *radicis-lycopersici* has not been demonstrated in nature and if heterokaryons form at all, they are likely to be transient (Di Primo *et al.* 2001 Di Primo *et al.* 2001).

It is also not known whether heterokaryons of *B. cinerea* occur in nature. There is evidence that a vegetative incompatibility system may be operating in *B. cinerea* (Delcán and Melgarejo 2002; Fournier *et al.* 2003; Weeds *et al.* 1998). Vegetative incompatibility reduces the expected degree of heterokaryosis and increases speculation that the diversity in *B. cinerea* may not be due to heterokaryosis. A system of vegetative incompatibility in *B. cinerea* or an absence of heterokaryosis in nature, or heterokaryosis that results only in transient heterokaryons, all place restrictions on the development of genetic variability. Alternative mechanisms are required to explain the degree of diversity that exists in *B. cinerea*.

It has been demonstrated that partial heterokaryon-incompatibility reactions can occur in fungi, ie. *Aspergillus nidulans* (Eidam) Winter, and that partial heterokaryon incompatibility is not an absolute barrier to gene flow (Coenen *et al.* 1994). Harman *et al.* (1998) proposed a mechanism to explain the diversity in *Trichoderma* spp. in the absence of heterokaryosis following an anastomosis event. The mechanism is referred to as interstrain gene transfer and proposes that only small pieces of DNA move from 1 genome to the other following anastomosis and these pieces are incorporated into the genome of the recipient strain (Harman *et al.* 1998).

Conidia of *B. cinerea* can contain 3 to 18 nuclei (Epton and Richmond 1980) and can be considered to represent a colony rather than an individual. They cannot form a 'pure strain' unless all the nuclei are identical (Lorbeer 1980). Hyphal cells have been reported to contain in excess of 100 nuclei (Lorbeer 1980). This multinucleate condition alone provides an explanation of a high degree of diversity based on differential gene expression that could occur among the various nuclei in cells and could give rise to many permutations of expression even within a population. If intergene transfer exists it would allow for interstrain exchange of DNA, even if only at a lesser rate than is possible under heterokaryosis. Clearly, there is much more work required in order to understand the genetic basis for the level of diversity present in *B. cinerea*.

2.5.4 Mechanisms of pathogenicity and host specificity

In order to colonize and parasitize a plant, pathogens must solve 2 problems, the first is to reach the interior of the plant and the second is to establish there in order to exploit the plant (Agrios 1997; Prell and Day 2001). Pathogenicity genes are responsible for the ability of pathogens to invade and utilize the plant and can be quite varied as they provide for many single steps such as penetration, formation of structures required to colonize the plant and establishing the necrotrophic or biotrophic modes of pathogenesis (Agrios 1997; Prell and Day 2001). Most necrotrophic pathogens first kill their host cells by excreting toxins and exoenzymes and all nectrotrophic fungi require the concerted action of many pathogenecity genes for successful pathogenesis (Prell and Day 2001). Many of the pathogenicity genes of necrotrophs directly serve functions to overpower host plant defences (Prell and Day 2001). Necrotrophs only colonize the cells of their hosts after the cells have been killed and the onset of disease symptoms are rapid (Agrios 1997).

Biotrophic fungi establish within living host cells and exploit their hosts by forming haustoria between the cell wall and plasma membrane which then draws on nutrients in the plant cell protoplast (Agrios 1997; Prell and Day 2001). Severe disease symptoms develop only after extensive colonization of the host tissue occurs. There is a highly specific interaction between biotrophs and their host plants in the recognition between pathogen and host and in establishing and maintaining parasitism with minimum disturbance of the plant (Agrios 1997; Prell and Day 2001). Biotrophic plant pathogenic fungi also rely on the action of many genes for successful pathogenesis and the actions of these genes are more specialized than in necrotrophs (Agrios 1997; Prell and Day 2001). In general biotrophs have a narrower host range where necrotrophs have a broad host range (Agrios 1997; Prell and Day 2001). Necrotrophs are prolific toxin producers and their enzymes have strong activity against the host plant tissue while biotrophs seldom produce toxins and their secreted enzymes have low activity against the host plant (Prell and Day 2001).

The differences in the nature of pathogencity between biotrophs and nectrotrophs is strongly linked to the likelihood of host specificity. The development of host specificity is not likely in necrotrophs and almost certain in biotrophs. It is unlikely that *B. cinerea*, as a genus, will develop host specificity as long as it remains exclusively necrotrophic.

The defence responses of plants are also genetically determined and are a result of the actions of a large number of genes (Devoto *et al.* 1997; Geffroy *et al.* 1999; Prell and Day 2001). Plant processes that prevent colonization by pathogens can be grouped into first level defences or basic resistance and second level defences that may be initiated only after basic resistance has been overcome. Second level defences are generally considered to be pathogen specific, often directed at a single race of the pathogen (Prell and Day 2001). In this context the second level defences of host plant resistance have also been described as having 2 components, vertical or race-specific, resistance and horizontal, or non-race specific, resistance (Agrios 1997; Prell and Day 2001). Vertical resistance prevents parasitism by 1 race of 1 pathogen and horizontal resistance prevents parasitism by all races of the 1 pathogen (Agrios 1997; Prell and Day 2001). Vertical resistance is considered to be relatively unstable as it may be overcome by a mutation of 1 gene in the pathogen where as horizontal resistance is more stable because more plant defence genes are involved (Agrios 1997; Prell and Day 2001). Race-specific resistance forms the basis of the gene-for-gene hypothesis of pathogen-host plant interaction.

Race-specific resistance in plants is primarily observed with biotrophs because they rely on a living host plant while second level resistance is rarely seen with necrotrophs which rapidly kill host plant tissue (Prell and Day 2001). It is currently understood that the development races of a pathogen is dependent on the existence of gene-for-gene systems in pathogenesis (Derso and Waller 2003).

Colletotrichum is a large genus of fungi that contains many plant pathogenic species causing anthracnose on a wide range of crop and ornamental plants (Perfect *et al.* 1999). Species of *Colletotrichum* have been commonly used to study fungal differentiation and fungal pathogen-host plant interactions as both biotrophic and necrotrophic strategies exist within this genus (Perfect *et al.* 1999). *Colletotrichum lindemuthianum* (Saccardo and Magnus) Briosi and Cavara, causes anthracnose of bean (*Phaseolus vulgaris* L.) and the *C. lindemuthianum* – bean interaction has been the focus of considerable study (Perfect *et al.* 1999).

Colletotrichum lindemuthianum employs a biotrophic strategy early in its pathogenesis on bean (Perfect *et al.* 1999) and is an example of a classic gene-for-gene pathosystem. Evolving races of *C. lindemuthianum* continue to overcome specific resistance genes in bean (Balardin and Kelly 1999). Balardin and Kelly (1999) investigated the interaction between 34 races of *C. lindemuthianum* and 62 modern bean cultivars and illustrated the extent and complexity of the diversity present in this pathosystem.

After extensive biotrophic colonization of the susceptible bean plant, *C. lindemuthianum* switches to necrotrophic development and host exploitation (Perfect *et al.* 1999). The onset of necrotrophy in the *C. lindemuthianum*-bean interaction is clearly linked to increased expression of cell wall degrading enzymes including endo-polygalactruonases and pectin-lyases (Perfect *et al.* 1999).

Botrytis cinerea is a necrotroph and the expression of endo-polygalacturonase and other cell wall degrading enzymes occurs during the early stages of infection (ten Have *et al.* 1998). No gene resistance genes are known to exist for *B. cinerea* in strawberry (Daugaard 2000) and it is unlikely that *B. cinerea* is involved in gene-for-gene pathogenesis with any of its hosts. As the development of races is unlikely in the absence of gene-for-gene pathogenesis a high degree of host specificity is not likely to be seen in *B. cinerea*.

It has been demonstrated that 6 endo-polygalactruonase genes are present in *B. cinerea* and that differential expression of these genes can vary with host (ten Have *et al.* 2001). It has also been shown that the number of genes in the genome of *Botrytis* spp. are not correlated with host range (Wubben *et al.* 1999). The requirement for a number of isozymes could be due to differences in substrate and environment and that the functional overlap of a number of genes would then not be a case of redundancy (ten Have *et al.* 2001).

Other research with single *B. cinerea* genes involved in pathogenicity and virulence has demonstrated that disruption of these individual genes may reduce pathogenicity and virulence of the fungus, but does not prevent disease development. Cutinase A was expressed by *B. cinerea* but not determined to be essential during infection of gerbera (*Gerbera jamesonii* H. Bolus ex J.D. Hook) and tomato (van Kan *et al.* 1997). The BAC gene important in the signaling process during infection, was also determined to be required for for full pathogenicty but not essential for successful pathogenesis (Klimpel *et al.* 2002). Disruption of the *B. cinerea* pectin methylesterase gene Bcpme1 also reduced virulence on several hosts but was not essential for pathogenesis (Valette-Collet 2003). In some cases single *B. cinerea* genes are required for pathogenesis. The *B. cinerea* mitogen-activated protein kinase gene BMP1 is essential for pathogenicity (Zheng *et al.* 2000).

It is probable that a number of clusters of genes encoding for various related pathogenesis enzymes and signaling processes exist in *B. cinerea* and that individual genes within these clusters could be expressed differentially depending on host and environment. Differential expression of these genes would allow for variability in pathogenesis and virulence between strains of *B. cinerea* within a population. It would explain the presence of strains virulent on a number of hosts, strains that display apparent host specialization, and strains that are weakly virulent on a number of hosts.

The results of the stem piece bioassay experiments clearly reveal the diversity in virulence of the *B. cinerea* strains examined against the 4 host species. This indicates that many genes are involved in pathogenesis. It is possible that all pathogenicity genes present in *B. cinerea* could be present in each strain and it is the expression of genes by different strains and in different hosts that varies. Wubben *et al.* (1999) characterized the 6 endo-polygalacturonase genes from *B. cinerea* and found that each member of the gene family was present in all the *B. cinerea* strains tested as well as being present in all of other *Botrytis* spp. strains examined.

The virulence of some of the strains is consistent across all plant hosts examined. Strains LF5 and BN3 are highly virulent on all 4 host species indicating a more potent expression of pathogenesis genes. Strains EP4 and TX2 are weakly virulent on all 4 host plant species and represent the other end of the spectrum of pathogenesis gene expression.

Host specialization of strains is also evident as strain LF4 was highly virulent on tomato, cucumber and petunia, yet weakly virulent on geranium. Strain EO2 was virulent on cucumber, moderately virulent on tomato and weakly virulent on geranium and non-pathogenic on petunia. Variability is evident among strains collected from the same location, strain LF1, LF4, and LF5 were all highly virulent on tomato while strains LF2 and LF3 were moderately virulent on tomato. Strains K1 and K3 were weakly virulent on tomato while strains K2, K4, and K5 were moderately virulent on tomato. Strains of *B. cinerea* demonstrate some host specialization yet populations of *B. cinerea* are likely to contain and express the genetic capability to cause disease on a wide host range.

The implications for disease management is that the development of resistant cultivars for *B. cinerea* is unlikely and that crop rotation will continue to be an ineffective strategy for disease control of *B. cinerea*.

2.6 Conclusions

This study is the first to characterize the virulence of *B. cinerea* strains, collected from a wide variety of diseased host plant material, against tomato and other host plants as the basis for selecting a highly virulent strain for use in evaluating potential biological control agents. The results of this study demonstrate variability in pathogenicity and virulence among strains of *B. cinerea* collected from the same or different plant hosts at 1 location as well as between strains collected from different hosts and different locations.

This study also demonstrates that recovery of *B. cinerea* strains from diseased plant material in the field will likely yield pathogenic strains but does not provide any indication of relative virulence on the intended host plant or other host plants. Individual strains from the same population can vary in virulence and that populations between locations also vary in virulence. The capability to cause disease does not provide any indication of virulence on the intended host plant species. A screening process is required to characterize pathogenicity and virulence of field strains.

Considerable effort and expense is required in the commercialization of biological control products (Harman 2000) and ensuring that biological control candidates are selected using a selection process that include a highly virulent strain of the target pathogen is a prudent practice, if not a necessity. Biological control agents selected for further development based on promising performance against less virulent strains of the pathogen may not stand up as well under field conditions. Failure to obtain a registration for a biological control product represents a significant loss for the developing company. More importantly registration of biological control products that do not stand up well in the field can damage the reputation of biological control agents as a whole. The use of highly virulent strains of the target pathogen early in the selection process should improve the probability of developing a more effective biological control product. This study provides a method to select a highly virulent strain of *B. cinerea* for use as the target strain in the screening of potential biological control agents.

This study identified strains of *B. cinerea* that are highly virulent on a number of hosts. LF5 is representative of a virulent strain of *B. cinerea* causing disease in commercial tomato greenhouses in Alberta. Strain LF5 is also highly virulent on cucumber, petunia and geranium. The development of a successful biological control agent is dependent on the selection process. An important consideration of the selection process is the environment in which the biological control agent is expected to perform. This includes the intended crop and physical environment in which the crop is grown as well as the pathogen. A more rigorous selection process can be established if a highly virulent strain of the pathogen is identified and selected for use. *Botrytis cinerea* strain LF5 was selected for use as the target strain of the development of a biological control for *B. cinerea* in greenhouse tomato.

2.7 Tables and figures

Table 2-1. *Botrytis cinerea* strains investigated in this study

Source ID	Source	Strain ID	Host Species	Location CANADA, Alberta
ST	Greenhouse	ST1	*Impatiens wallerana* Hook.	Medicine Hat
		ST2		
		ST3		
		ST4		
		ST5		
SB	Greenhouse	SB1	*Fragaria* × *ananassa* Duch.	Brooks
		SB2		
		SB3		
		SB4		
		SB5		
SD	Greenhouse	SD1	*Pelargonium* × *hortorum* L.H. Bailey	Rocky Mountain House
		SD2		
		SD3		
		SD4		
		SD4		
PF	Greenhouse	PF1	*Lactuca sativa* L.	Lyalta
		PF2		
		PF3		
		PF4		
		PF5		
MK	Greenhouse	MK1	*Pelargonium* × *hortorum* L.H. Bailey	Gull Lake
		MK2		
		MK3		
		MK4		
		MK5		
HR	Greenhouse	HR1	*Begonia* × *semperflorens –cultorum* Hort.	Brooks
		HR2	*Begonia* × *semperflorens –cultorum* Hort.	
		HR3	*Pelargonium* × *hortorum* L.H. Bailey	
		HR4	*Pelargonium* × *hortorum* L.H. Bailey	
		HR5	*Fuchsia* × *hybrida* Hort. Ex Vilm.	
BN	Greenhouse	BN1	*Lycopersicon esculentum* Mill.	Millerville
		BN2		
		BN3		
		BN4		
		BN5		
TM	Greenhouse	TM1	*Lycopersicon esculentum* Mill.	Brooks
		TM2		
		TM3		
		TM4		
		TM5		

VW	Greenhouse	VW1	*Lycopersicon esculentum* Mill.	Medicine Hat
		VW2		
		VW3		
		VW4		
		Vw5		
LF	Greenhouse	LF1	*Lycopersicon esculentum* Mill.	Strathmore
		LF2		
		LF3		
		LF4		
		LF5		
EC	Field	EC1	*Echinacea angustifolia* DC.	Brooks
		EC2		
		EC3		
		EC4		
		EC5		
PR	Greenhouse	PR1	*Pelargonium* × *hortorum* L.H. Bailey	Rocky Mountain House
		PR2		
		PR3		
		PR4		
		PR5		
WL	Greenhouse	WL1	*Pelargonium* × *hortorum* L.H. Bailey	Edmonton
		WL2	*Petunia* × *hybrida* Hort. Vilm.–Andr.	
		WL3	*Pelargonium* × *hortorum* L.H. Bailey	
		WL4	*Impatiens wallerana* Hook.	
		WL5	*Lactuca sativa* L.	
K	Greenhouse	K1	*Petunia* × *hybrida* Hort. Vilm.–Andr.	Edmonton
		K2	*Petunia* × *hybrida* Hort. Vilm.–Andr.	
		K3	*Ageratum houstonianum* Mill.	
		K4	*Impatiens wallerana* Hook.	
		K5	*Pelargonium* × *hortorum* L.H. Bailey	
WD	Greenhouse	WD1	*Tagetes erecta* L.	Medicine Hat
		WD2	*Lycopersicon esculentum* Mill.	
		WD3	*Tagetes erecta* L.	
		WD4	*Lycopersicon esculentum* Mill.	
		WD5	*Lycopersicon esculentum* Mill.	
MV	Greenhouse	MV1	*Petunia* × *hybrida* Hort. Vilm.–Andr.	Morinville
		MV2	*Lactuca sativa* L.	
		MV3	*Lactuca sativa* L.	
		MV4	*Petunia* × *hybrida* Hort. Vilm.–Andr.	
		MV5	*Petunia* × *hybrida* Hort. Vilm.–Andr.	
RH	Greenhouse	RH1	*Pelargonium* × *hortorum* L.H. Bailey	Coaldale
		RH2		
		RH3		
		RH4		
		RH5		

TS	Greenhouse	TS1	*Lycopersicon esculentum* Mill.	Redcliff
		TS2		
		TS3		
		TS4		
		TS5		
BA	Greenhouse	BA1	*Lycopersicon esculentum* Mill.	Gull Lake
		BA2		
		BA3		
		BA4		
		BA5		
EO	Greenhouse	EO1	*Cucumis sativus* L.	Bragg Creek
		EO2	*Cucumis sativus* L.	
		EO3	*Cucumis sativus* L.	
		EO4	*Pelargonium* × *hortorum* L.H. Bailey	
		EO5	*Pelargonium* × *hortorum* L.H. Bailey	
BL	Field	BL1	*Fragaria* × *ananassa* Duch.	Brooks
		BL2		
		BL3		
		BL4		
		BL5		
AL	Field	AL1	*Medicago sativa* L.	Brooks
		AL2		
		AL3		
		AL4		
		AL5		
SC	Greenhouse	SC1	*Limonium latifolium* O. Kuntz × *L. bellidifolium* (Gouan) Dumort	Brooks
		SC2		
		SC3		
		SC4		
		SC5		
HN	Field	HN1	*Galeopsis tetrahit* L.	Edmonton
		HN2		
		HN3		
		HN4		
		HN5		
JP	Field	JP1	*Paeonia lactiflora* Pal.	Jasper
		JP2		
		JP3		
		JP4		
		JP5		
BT	Greenhouse	BT1	*Lycopersicon esculentum* Mill.	Strathmore
		BT2		
		BT3		
		BT4		
		BT5		

EP	Field	EP1	*Pisum satvum* L.	Edmonton
		EP2		
		EP3		
		EP4		
		EP5		
TX	Field	TX1	*Linaria vulgaris* Mill.	Edmonton
		TX2		
		TX3		
		TX4		
		TX5		
CA	Field	CA1	*Brassica rapa* L.	Westlock
		CA2		
		CA3		
		CA4		
		CA5		
LC	Field	LC1	*Lactuca sativa* L.	Brooks
		LC2		
		LC3		
		LC4		
		LC5		
PT	Greenhouse	PT1	*Phaseolus vulgaris* L.	Brooks
		PT2		
		PT3		
		PT4		
		PT5		
SA	Greenhouse	SA1	*Petunia* × *hybrida* Hort. Vilm.–Andr.	Edmonton
		SA2		
		SA3		
		SA4		
		SA5		

Table 2-2. Variability in culture characteristics of the 160 strains of *Botrytis cinerea* grown on PDA and assessed on the basis of mycelium colour, sclerotial production and production of conidia. Cultures assessed at 1 week of age.

Source ID	**Strain ID**	**Mycelium Colour**	**Sclerotia**	**Conidia Production (Hansen designation)**
ST	ST1	Light	No	C
	ST2	Light	No	C
	ST3	Light	No	C
	ST4	Light	No	C
	ST5	Light	No	C
SB	SB1	Light	No	C/M
	SB2	Light	No	C/M
	SB3	Light	No	C/M
	SB4	Light	No	C/M
	SB5	Light	No	C/M
SD	SD1	Light	Yes	C/M
	SD2	Light	Yes	C/M
	SD3	Light	No	C/M
	SD4	Light	No	C/M
	SD4	Light	No	C/M
PF	PF1	Light	No	C/M
	PF2	Light	No	C/M
	PF3	Light	No	C/M
	PF4	Light	No	C/M
	PF5	Light	No	C/M
MK	MK1	Light	No	C
	MK2	Light	No	C
	MK3	Light	Yes	C
	MK4	Light	No	C
	MK5	Light	Yes	C/M
HR	HR1	Light	No	C
	HR2	Light	No	C
	HR3	Light	No	C
	HR4	Light	No	C
	HR5	Light	No	C
BN	BN1	Light	No	C
	BN2	Light	No	C
	BN3	Light	No	C/M
	BN4	Light	No	C
	BN5	Light	No	C

TM	TM1	Light	Yes	C/M
	TM2	Light	Yes	C/M
	TM3	Light	Yes	C/M
	TM4	Light	Yes	C/M
	TM5	Light	Yes	C/M
VW	VW1	Light	No	C/M
	VW2	Light	No	C/M
	VW3	Light	No	C/M
	VW4	Light	No	C/M
	VW5	Light	No	C/M
LF	LF1	Light	Yes	C
	LF2	Light	Yes	C/M
	LF3	Light	Yes	C/M
	LF4	Light	Yes	C/M
	LF5	Light	Yes	C/M
EC	EC1	Light	No	C/M
	EC2	Light	Yes	C/M
	EC3	Light	Yes	C/M
	EC4	Light	No	C/M
	EC5	Light	No	M
PR	PR1	Light	No	C/M
	PR2	Light	No	C/M
	PR3	Light	No	C/M
	PR4	Light	No	C/M
	PR5	Light	No	C/M
WL	WL1	Light	Yes	C/M
	WL2	Light	Yes	C/M
	WL3	Light	Yes	C/M
	WL4	Light	Yes	C/M
	WL5	Light	No	M
K	K1	Light	Yes	C/M
	K2	Light	No	C/M
	K3	Light	Yes	C
	K4	Light	Yes	C/M
	K5	Light	No	C
WD	WD1	Light	No	C/M
	WD2	Light	No	C/M
	WD3	Light	No	C/M
	WD4	Light	No	C/M
	WD5	Light	No	C/M
MV	MV1	Light	No	C
	MV2	Light	Yes	C/M
	MV3	Light	Yes	C/M
	MV4	Light	Yes	C/M
	MV5	Light	Yes	C/M

RH	RH1	Light	No	C/M
	RH2	Light	No	C/M
	RH3	Light	No	C/M
	RH4	Light	No	C/M
	RH5	Light	No	C/M
TS	TS1	Light	No	C
	TS2	Light	No	C
	TS3	Light	No	C
	TS4	Light	No	C
	TS5	Light	No	C
BA	BA1	Light	No	C
	BA2	Light	No	C
	BA3	Light	No	C
	BA4	Light	No	C
	BA5	Light	No	C
EO	EO1	Light	Yes	C/M
	EO2	Dark	No	C/M
	EO3	Light	No	C/M
	EO4	Light	No	C/M
	EO5	Light	Yes	C/M
BL	BL1	Light	No	C/M
	BL2	Light	No	C/M
	BL3	Light	No	C/M
	BL4	Light	No	C/M
	BL5	Light	No	C/M
AL	AL1	Dark	No	C/M
	AL2	Dark	No	C/M
	AL3	Dark	No	C/M
	AL4	Dark	No	C/M
	AL5	Dark	No	C
SC	SC1	Light	No	C
	SC2	Light	No	C
	SC3	Light	No	C
	SC4	Light	No	C
	SC5	Light	No	C
HN	HN1	Light	No	C/M
	HN2	Light	No	C/M
	HN3	Light	No	C/M
	HN4	Light	No	C/M
	HN5	Light	No	C/M
JP	JP1	Light	Yes	M
	JP2	Light	Yes	M
	JP3	Light	Yes	M
	JP4	Light	Yes	M
	JP5	Light	Yes	M

BT	BT1	Light	No	C/M
	BT2	Light	No	C/M
	BT3	Light	No	C/M
	BT4	Light	No	C/M
	BT5	Light	No	C/M
EP	EP1	Light	Yes	C/M
	EP2	Light	Yes	C/M
	EP3	Light	Yes	C/M
	EP4	Light	Yes	C/M
	EP5	Light	Yes	C/M
TX	TX1	Light	Yes	M
	TX2	Light	Yes	M
	TX3	Light	Yes	M
	TX4	Light	Yes	M
	TX5	Light	Yes	M
CA	CA1	Light	Yes	C/M
	CA2	Light	Yes	C/M
	CA3	Dark	No	M
	CA4	Light	Yes	C/M
	CA5	Dark	No	C
LC	LC1	Light	Yes	M
	LC2	Light	Yes	M
	LC3	Light	Yes	M
	LC4	Light	Yes	M
	LC5	Light	Yes	M
PT	PT1	Light	No	C/M
	PT2	Light	No	C/M
	PT3	Light	No	C/M
	PT4	Light	No	C/M
	PT5	Light	No	C/M
SA	SA1	Dark	Yes	C/M
	SA2	Dark	No	M
	SA3	Dark	Yes	C
	SA4	Dark	No	M
	SA5	Dark	Yes	C/M

Table 2-3. Morphological characteristics of 3 representative, 1 week-old, cultures of *Botrytis cinerea* strains on PDA exhibiting differential conidia production corresponding to three categories C – heavy conidial production, C/M – intermediate conidial production and M – low conidial production

Strain	**Conidial production category**	**Condial dimensions***	**Conidiophore diameter***
LF1	C	8.0-16.6x 6.4-9.6 µm	8.0-17.6 µm
LF5	C/M	8.0-14.4 x 6.5-9.6 µm	12.8-19.2 µm
TX1	M	8.0-14.4 x 6.4-11.2 µm	12.8-17.6 µm

* Range of measurements obtained from 10 conidia and conidiophores

Table 2-4. Mean lesion length on tomato stem pieces (*Lycopersicon esculentum* Mill) (cv. Quest, n = 8) caused by strains of *Botrytis cinerea* 1 week after inoculation.

Strain	**Source Host**	**Lesion length (mm)**
LF5	*Lycopersicon esculentum* Mill.	22.8
LF1	*Lycopersicon esculentum* Mill.	20.9
LF4	*Lycopersicon esculentum* Mill.	19.0
BN3	*Lycopersicon esculentum* Mill.	18.6
BT2	*Lycopersicon esculentum* Mill.	18.0
BA4	*Lycopersicon esculentum* Mill.	17.6
BA1	*Lycopersicon esculentum* Mill.	17.6
BT1	*Lycopersicon esculentum* Mill.	17.6
SB4	*Fragaria × ananassa* Duch.	17.5
BT3	*Lycopersicon esculentum* Mill.	16.8
K5	*Pelargonium × hortorum* L.H. Bailey	16.5
SB1	*Fragaria × ananassa* Duch.	16.1
MV5	*Petunia × hybrida* Hort. Vilm.–Andr.	15.6
BT5	*Lycopersicon esculentum* Mill.	15.4
ST1	*Impatiens wallerana* Hook.	14.0
VW4	*Lycopersicon esculentum* Mill.	13.9
VW5	*Lycopersicon esculentum* Mill.	13.8
EO2	*Cucumis sativus* L.	13.8
AL1	*Medicago sativa* L.	13.8
MK3	*Pelargonium × hortorum* L.H. Bailey	13.6
ST4	*Impatiens wallerana* Hook.	13.5
VW2	*Lycopersicon esculentum* Mill.	13.5
PA2	*Petunia × hybrida* Hort. Vilm.–Andr.	13.2
LF3	*Lycopersicon esculentum* Mill.	13.2
EO1	*Cucumis sativus* L.	13.1
LF2	*Lycopersicon esculentum* Mill.	13.0
BA3	*Lycopersicon esculentum* Mill.	12.9
BT4	*Lycopersicon esculentum* Mill.	12.9
EC2	*Echinacea angustifolia* DC.	12.9
K4	*Impatiens wallerana* Hook.	12.8
WD4	*Lycopersicon esculentum* Mill.	12.8
BN5	*Lycopersicon esculentum* Mill.	12.5
WL5	*Lactuca sativa* L.	12.4
HN4	*Galeopsis tetrahit* L.	12.1
ST5	*Impatiens wallerana* Hook.	12.1
ST2	*Impatiens wallerana* Hook.	12.1
LC5	*Lactuca sativa* L.	11.9
MK1	*Pelargonium × horterum* L.H. Bailey	11.8
PR3	*Pelargonium × horterum* L.H. Bailey	11.8
MK2	*Pelargonium × horterum* L.H. Bailey	11.6
ST3	*Impatiens wallerana* Hook.	11.5
BN1	*Lycopersicon esculentum* Mill.	11.5

HN3	*Galeopsis tetrahit* L.	11.5
WD2	*Lycopersicon esculentum* Mill.	11.2
WL4	*Impatiens wallerana* Hook.	11.2
SD2	*Pelargonium* × *hortorum* L.H. Bailey	11.1
K2	*Petunia* × *hybrida* Hort. Vilm.–Andr.	11.0
EC5	*Echinacea angustifolia* DC.	11.0
MV4	*Petunia* × *hybrida* Hort. Vilm.–Andr.	10.9
TS3	*Lycopersicon esculentum* Mill.	10.9
SD1	*Pelargonium* × *hortorum* L.H. Bailey	10.8
BA5	*Lycopersicon esculentum* Mill.	10.8
EO3	*Cucumis sativus* L.	10.8
HN2	*Galeopsis tetrahit* L.	10.6
PR4	*Pelargonium* × *hortorum* L.H. Bailey	10.6
BN4	*Lycopersicon esculentum* Mill.	10.5
PT4	*Phaseolus vulgaris* L.	10.5
PF4	*Lactuca sativa* L.	10.4
RH3	*Pelargonium* × *hortorum* L.H. Bailey	10.4
PF5	*Lactuca sativa* L.	10.4
BA2	*Lycopersicon esculentum* Mill.	10.4
AL3	*Medicago sativa* L.	10.4
PF2	*Lactuca sativa* L.	10.2
BN2	*Lycopersicon esculentum* Mill.	10.2
SD3	*Pelargonium* × *hortorum* L.H. Bailey	10.1
SD5	*Pelargonium* × *hortorum* L.H. Bailey	10.1
TS4	*Lycopersicon esculentum* Mill.	10.0
TS5	*Lycopersicon esculentum* Mill.	10.0
WL1	*Pelargonium* × *hortorum* L.H. Bailey	10.0
K3	*Ageratum houstonianum* Mill.	9.9
AL2	*Medicago sativa* L.	9.8
SC2	*Limonium latifolium* O. Kuntz × *L. bellidifolium* (Gouan) Dumort	9.8
TS2	*Lycopersicon esculentum* Mill.	9.8
TS1	*Lycopersicon esculentum* Mill.	9.5
MV2	*Lactuca sativa* L.	9.5
TM4	*Lycopersicon esculentum* Mill.	9.5
WD1	*Tagetes erecta* L.	9.4
HR4	*Pelargonium* × *hortorum* L.H. Bailey	9.2
PR5	*Pelargonium* × *hortorum* L.H. Bailey	9.1
HN5	*Galeopsis tetrahit* L.	9.1
EO4	*Pelargonium* × *hortorum* L.H. Bailey	9.1
SB5	*Fragaria* × *ananassa* Duch.	9.0
RH5	*Pelargonium* × *hortorum* L.H. Bailey	9.0
MK5	*Pelargonium* × *hortorum* L.H. Bailey	8.9
EC3	*Echinacea angustifolia* DC.	8.9
RH4	*Pelargonium* × *hortorum* L.H. Bailey	8.6
MK4	*Pelargonium* × *hortorum* L.H. Bailey	8.5
EC4	*Echinacea angustifolia* DC.	8.5
VW1	*Lycopersicon esculentum* Mill.	8.5

SD4	*Pelargonium* × *hortorum* L.H. Bailey	8.4
MV3	*Lactuca sativa* L.	8.4
HR1	*Begonia* × *semperflorens –cultorum* Hort.	8.4
WL2	*Petunia* × *hybrida* Hort. Vilm.–Andr.	8.2
RH2	*Pelargonium* × *hortorum* L.H. Bailey	8.2
EC1	*Echinacea angustifolia* DC.	8.1
SC5	*Limonium latifolium* O. Kuntz × *L. bellidifolium* (Gouan) Dumort	8.1
PA4	*Petunia* × *hybrida* Hort. Vilm.–Andr.	8.1
EP2	*Pisum satvum* L.	8.1
SC1	*Limonium latifolium* O. Kuntz × *L. bellidifolium* (Gouan) Dumort	8.0
CA2	*Brassica rapa* L.	8.0
CA5	*Brassica rapa* L.	7.9
TM2	*Lycopersicon esculentum* Mill.	7.7
HR5	*Fuchsia* × *hybrida* Hort. Ex Vilm.	7.6
VW3	*Lycopersicon esculentum* Mill.	7.6
HR2	*Begonia* × *semperflorens –cultorum* Hort.	7.6
PR1	*Pelargonium* × *hortorum* L.H. Bailey	7.5
SC4	*Limonium latifolium* O. Kuntz × *L. bellidifolium* (Gouan) Dumort	7.5
BL2	*Fragaria* × *ananassa* Duch.	7.4
PR2	*Pelargonium* × *hortorum* L.H. Bailey	7.2
JP2	*Paeonia lactiflora* Pal.	7.2
JP3	*Paeonia lactiflora* Pal.	7.2
TM3	*Lycopersicon esculentum* Mill.	7.2
PT2	*Phaseolus vulgaris* L.	7.1
PT3	*Phaseolus vulgaris* L.	7.0
CA1	*Brassica rapa* L.	7.0
HN1	*Galeopsis tetrahit* L.	7.0
PT1	*Phaseolus vulgaris* L.	7.0
SB3	*Fragaria* × *ananassa* Duch.	7.0
EP3	*Pisum satvum* L.	6.9
HR3	*Pelargonium* × *hortorum* L.H. Bailey	6.7
AL4	*Medicago sativa* L.	6.7
PF3	*Lactuca sativa* L.	6.7
PT5	*Phaseolus vulgaris* L.	6.7
AL5	*Medicago sativa* L.	6.7
CA3	*Brassica rapa* L.	6.6
PA5	*Petunia* × *hybrida* Hort. Vilm.–Andr.	6.6
LC1	*Lactuca sativa* L.	6.4
PA1	*Petunia* × *hybrida* Hort. Vilm.–Andr.	6.2
JP4	*Paeonia lactiflora* Pal.	6.1
TM5	*Lycopersicon esculentum* Mill.	6.1
LC2	*Lactuca sativa* L.	6.0
SC3	*Limonium latifolium* O. Kuntz × *L. bellidifolium* (Gouan) Dumort	5.8

JP1	*Paeonia lactiflora* Pal.	5.8
WL3	*Pelargonium* × *hortorum* L.H. Bailey	5.8
MV1	*Petunia* × *hybrida* Hort. Vilm.–Andr.	5.8
LC4	*Lactuca sativa* L.	5.7
SB2	*Fragaria* × *ananassa* Duch.	5.6
BL4	*Fragaria* × *ananassa* Duch.	5.6
EP1	*Pisum satvum* L.	5.5
EO5	*Pelargonium* × *hortorum* L.H. Bailey	5.4
JP5	*Paeonia lactiflora* Pal.	5.4
PA3	*Petunia* × *hybrida* Hort. Vilm.–Andr.	5.0
BL1	*Fragaria* × *ananassa* Duch.	4.8
K1	*Petunia* × *hybrida* Hort. Vilm.–Andr.	4.8
BL3	*Fragaria* × *ananassa* Duch.	4.7
PF1	*Lactuca sativa* L.	4.7
RH1	*Pelargonium* × *hortorum* L.H. Bailey	4.6
EP4	*Pisum satvum* L.	4.2
WD5	*Lycopersicon esculentum* Mill.	4.0
LC3	*Lactuca sativa* L.	3.8
WD3	*Tagetes erecta* L.	3.6
EP5	*Pisum satvum* L.	3.4
TX1	*Linaria vulgaris* Mill.	3.4
BL5	*Fragaria* × *ananassa* Duch.	3.4
TM1	*Lycopersicon esculentum* Mill.	3.2
TX4	*Linaria vulgaris* Mill.	2.6
TX2	*Linaria vulgaris* Mill.	2.5
CA4	*Brassica rapa* L.	2.5
TX5	*Linaria vulgaris* Mill.	2.2
TX3	*Linaria vulgaris* Mill.	2.1
Control		0.1

ANOVA Table

Source	D.F.	Type III S.S.	Mean Square	F value	Pr. > F
Date	1	21574.00	21574	252.13	<0.0001
Strain	160	19862.00	124.12	1.45	0.0096
Date*Strain	160	13694.00	85.59	3.30	<0.0001
Error	963	24992.00	25.95		

Table 2-5. Mean lesion length on cucumber stem pieces (*Cucumis sativus* L.) (cv. Flamingo, n = 4) caused by strains of *Botrytis cinerea* 1 week after inoculation.

Strain	Source host	Lesion length (mm)
LF4	*Lycopersicon esculentum* Mill.	24.8
SB4	*Fragaria* × *ananassa* Duch.	23.5
LF1	*Lycopersicon esculentum* Mill.	23.2
MV4	*Petunia* × *hybrida* Hort. Vilm.–Andr.	22.2
PF2	*Lactuca sativa* L.	21.8
AL2	*Medicago sativa* L.	21.0
LF5	*Lycopersicon esculentum* Mill.	19.8
BN3	*Lycopersicon esculentum* Mill.	19.0
PR2	*Lactuca sativa* L.	19.0
EO3	*Cucumis sativus* L.	18.0
EC1	*Echinacea angustifolia* DC.	17.0
WL1	*Pelargonium* × *hortorum* L.H. Bailey	17.0
EO1	*Cucumis sativus* L.	16.8
RH1	*Pelargonium* × *hortorum* L.H. Bailey	16.5
EO2	*Cucumis sativus* L.	16.5
SD5	*Pelargonium* × *hortorum* L.H. Bailey	16.2
LC5	*Lactuca sativa* L.	16.0
BT1	*Lycopersicon esculentum* Mill.	16.0
PT4	*Phaseolus vulgaris* L.	15.2
K2	*Petunia* × *hybrida* Hort. Vilm.–Andr.	14.2
BA1	*Lycopersicon esculentum* Mill.	13.2
CA1	*Brassica rapa* L.	12.5
HR4	*Pelargonium* × *hortorum* L.H. Bailey	12.2
JP4	*Paeonia lactiflora* Pal.	11.8
ST4	*Impatiens wallerana* Hook.	11.5
TS2	*Lycopersicon esculentum* Mill.	11.5
MK4	*Pelargonium* × *hortorum* L.H. Bailey	10.5
PA3	*Petunia* × *hybrida* Hort. Vilm.–Andr.	10.0
WD1	*Tagetes erecta* L.	10.0
EP4	*Pisum satvum* L.	6.2
SC5	*Limonium latifolium* O. Kuntz × *L. bellidifolium* (Gouan) Dumort	3.2
TX2	*Linaria vulgaris* Mill.	1.8
HN3	*Galeopsis tetrahit* L.	1.5
VW4	*Lycopersicon esculentum* Mill.	1.3
TM1	*Lycopersicon esculentum* Mill.	1.0
Control		0.0

ANOVA Table

Source	D.F.	Type III S.S.	Mean Square	F value	Pr. > F
Strain	35	6612.52	188.93	4.41	<0.0001
Error	107	4585.92	42.86		

Table 2-6. Mean lesion length on geranium stem pieces (*Pelargonium* × *hortorum* L.H. Bailey) (cv. Kim, n = 4) colonized by strains of *Botrytis cinerea* 1 week after inoculation.

Strain	**Source host**	**Lesion length (mm)**
SB4	*Fragaria* × *ananassa* Duch.	21.5
BN3	*Lycopersicon esculentum* Mill.	20.0
ST4	*Impatiens wallerana* Hook.	19.5
LF5	*Lycopersicon esculentum* Mill.	19.2
BT1	*Lycopersicon esculentum* Mill.	19.0
BA1	*Lycopersicon esculentum* Mill.	18.2
EC1	*Echinacea angustifolia* DC.	17.5
PF2	*Lactuca sativa* L.	17.0
HR4	*Pelargonium* × *hortorum* L.H. Bailey	15.5
EO1	*Cucumis sativus* L.	15.0
MK4	*Pelargonium* × *hortorum* L.H. Bailey	15.0
JP4	*Paeonia lactiflora* Pal.	14.5
PA3	*Petunia* × *hybrida* Hort. Vilm.–Andr.	14.5
AL2	*Medicago sativa* L.	14.5
LF1	*Lycopersicon esculentum* Mill.	14.2
CA1	*Brassica rapa* L.	14.2
PR2	*Lactuca sativa* L.	14.2
HN3	*Galeopsis tetrahit* L.	13.8
VW4	*Lycopersicon esculentum* Mill.	13.7
EO3	*Cucumis sativus* L.	13.2
TS2	*Lycopersicon esculentum* Mill.	13.0
WL1	*Pelargonium* × *hortorum* L.H. Bailey	11.8
LC5	*Lactuca sativa* L.	11.8
PT4	*Phaseolus vulgaris* L.	11.5
RH1	*Pelargonium* × *hortorum* L.H. Bailey	10.8
K2	*Petunia* × *hybrida* Hort. Vilm.–Andr.	9.5
EO2	*Cucumis sativus* L.	9.2
LF4	*Lycopersicon esculentum* Mill.	7.5
WD1	*Tagetes erecta* L.	6.0
EP4	*Pisum satvum* L.	5.5
SD5	*Pelargonium* × *hortorum* L.H. Bailey	5.5
MV4	*Petunia* × *hybrida* Hort. Vilm.–Andr.	4.5
SC5	*Limonium latifolium* O. Kuntz × *L. bellidifolium* (Gouan) Dumort	4.2
TM1	*Lycopersicon esculentum* Mill.	1.5
TX2	*Linaria vulgaris* Mill.	1.5
Control		0.5

ANOVA Table

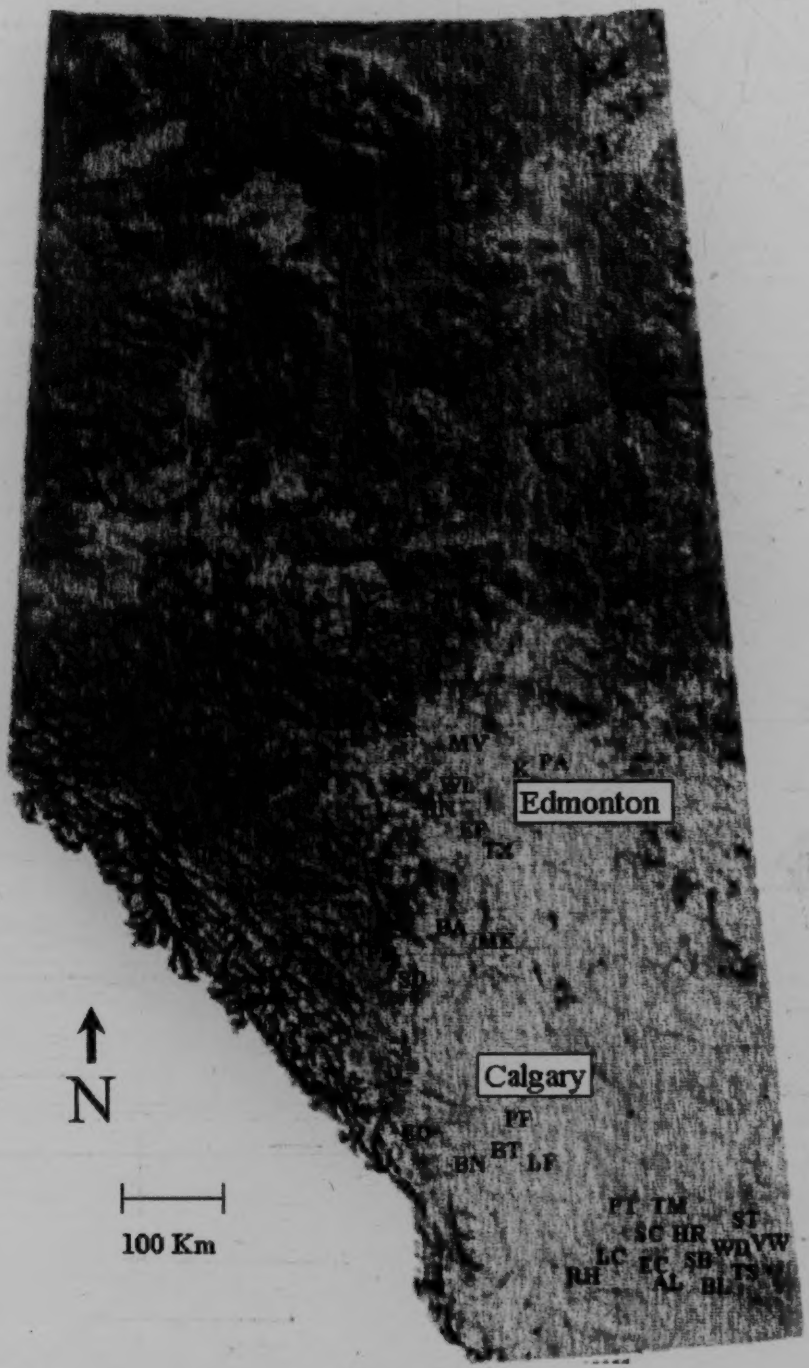
Source	D.F.	Type III S.S.	Mean Square	F value	Pr. > F
Strain	35	4410.96	126.03	4.42	<0.0001
Error	107	3051.67	28.52		

Table 2-7. Mean lesion length on petunia (*Petunia* x *hybrida* Hort. Vilm.-Andr.) (cv. Purple Wave, N = 4) stem pieces caused by strains of *Botrytis cinerea* 1 week after inoculation.

Strain	Source host	Mean stem lesion length (mm)
EO1	*Cucumis sativus* L.	22.8
TM1	*Lycopersicon esculentum* Mill.	22.5
BN3	*Lycopersicon esculentum* Mill.	22.0
LF5	*Lycopersicon esculentum* Mill	21.2
BT1	*Lycopersicon esculentum* Mill	19.8
LF4	*Lycopersicon esculentum* Mill.	18.5
PA3	*Petunia* × *hybrida* Hort. Vilm.–Andr.	18.0
PF2	*Lactuca sativa* L.	17.0
PT4	*Phaseolus vulgaris* L.	16.0
EC1	*Echinacea angustifolia* DC.	14.5
JP4	*Paeonia lactiflora* Pal.	14.5
ST4	*Impatiens wallerana* Hook.	14.2
LC5	*Lactuca sativa* L.	14.2
VW4	*Lycopersicon esculentum* Mill.	13.3
K2	*Petunia* × *hybrida* Hort. Vilm.–Andr.	12.0
SB4	*Fragaria* × *ananassa* Duch.	11.5
HN3	*Galeopsis tetrahit* L.	11.2
MK4	*Pelargonium* × *hortorum* L.H. Bailey	10.8
HR4	*Pelargonium* × *hortorum* L.H. Bailey	10.0
EO3	*Cucumis sativus* L.	9.8
AL2	*Medicago sativa* L.	9.2
SC5	*Limonium latifolium* O. Kuntz × *L. bellidifolium* (Gouan) Dumort	8.8
WL1	*Pelargonium* × *hortorum* L.H. Bailey	8.5
MV4	*Petunia* × *hybrida* Hort. Vilm.–Andr.	7.8
BA1	*Lycopersicon esculentum* Mill.	7.8
PR2	*Lactuca sativa* L.	7.5
RH1	*Pelargonium* × *hortorum* L.H. Bailey	7.2
WD1	*Tagetes erecta* L.	7.2
LF1	*Lycopersicon esculentum* Mill.	7.0
CA1	*Brassica rapa* L.	5.8
TX2	*Linaria vulgaris* Mill.	4.5
SD5	*Pelargonium* × *hortorum* L.H. Bailey	3.5
TS2	*Lycopersicon esculentum* Mill.	3.2
EO2	*Cucumis sativus* L.	1.8
EP4	*Pisum satvum* L.	1.2
Control		0.0

ANOVA Table

Source	D.F.	Type III S.S.	Mean Square	F value	Pr. > F
Strain	35	5482.22	156.63	1.67	<0.0242
Error	107	10043.00	93.86		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K
PA
WL
Edmonton
EP
TX
BA
MK
SD
Calgary
PF
ED
BT
BN
LF
N
100 Km
PT
TM
ST
SC
HR
WD
VW
LC
EC
SB
RH
AL
TS
BL

Figure 2-1. Collection locations of the *Botrytis cinerea* strains investigated. (Map courtesy of Alberta Agriculture, Food and Rural Development.)

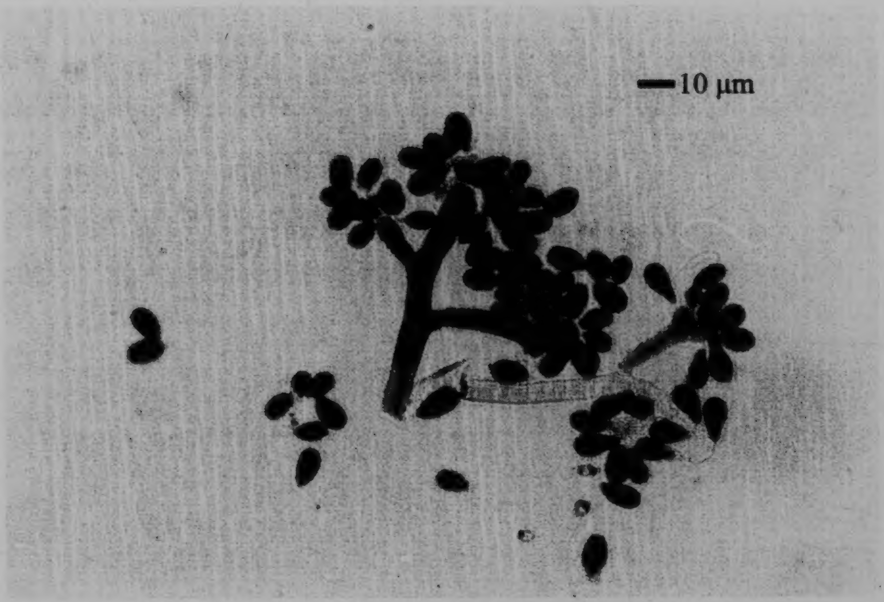

Figure 2-2. Light micrograph of acid fuchsin stained botryose clusters of *Botrytis cinerea* conidia formed at the tips of a branched conidiophore. From a 1-week old culture grown on PDA.

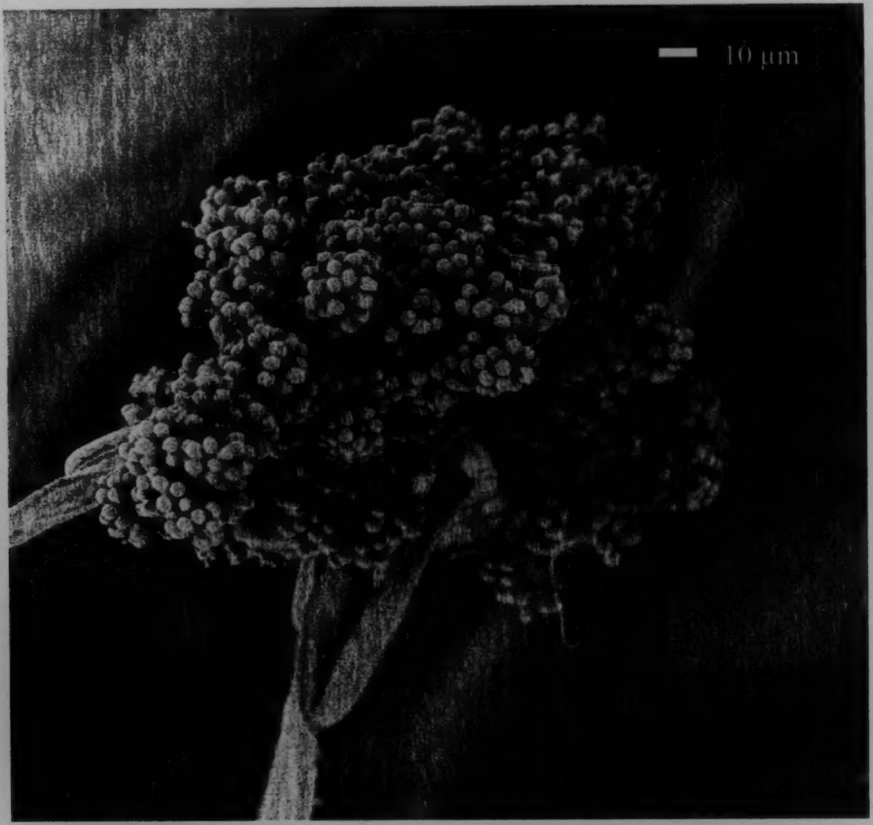

Figure 2-3. Scanning electron micrograph of botryose clusters of *Botrytis cinerea* conidia formed at the tips of a branched conidiophore. Courtesy of Dr. J.P. Tewari

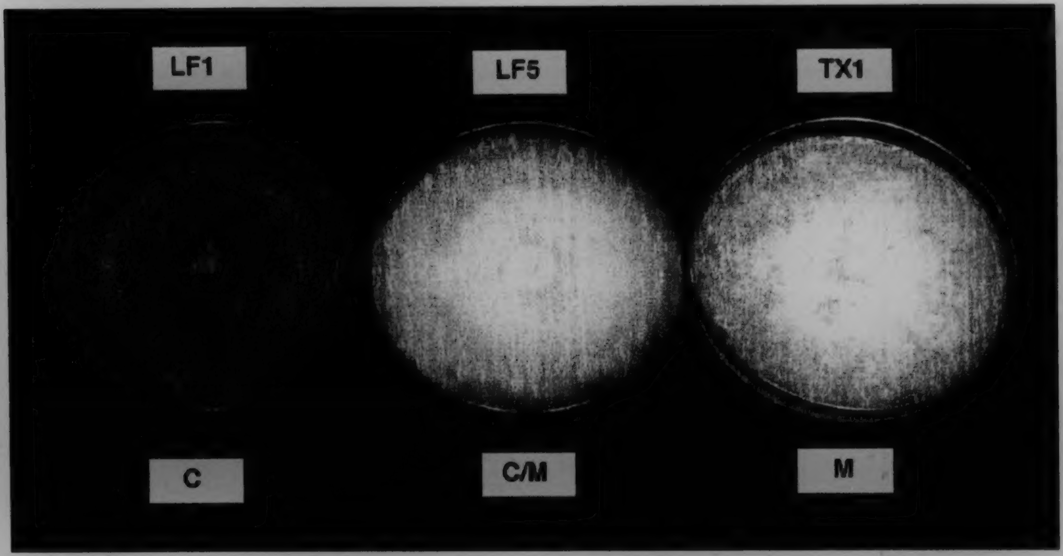

Figure 2-4. Representative 1-week old cultures of *Botrytis cinerea* strains exhibiting differential conidia production on PDA corresponding to the three categories C – heavy conidial production, C/M – intermediate conidial production and M – low conidial production. Represented respectively, from left to right, by strain LF1, LF5 and TX1.

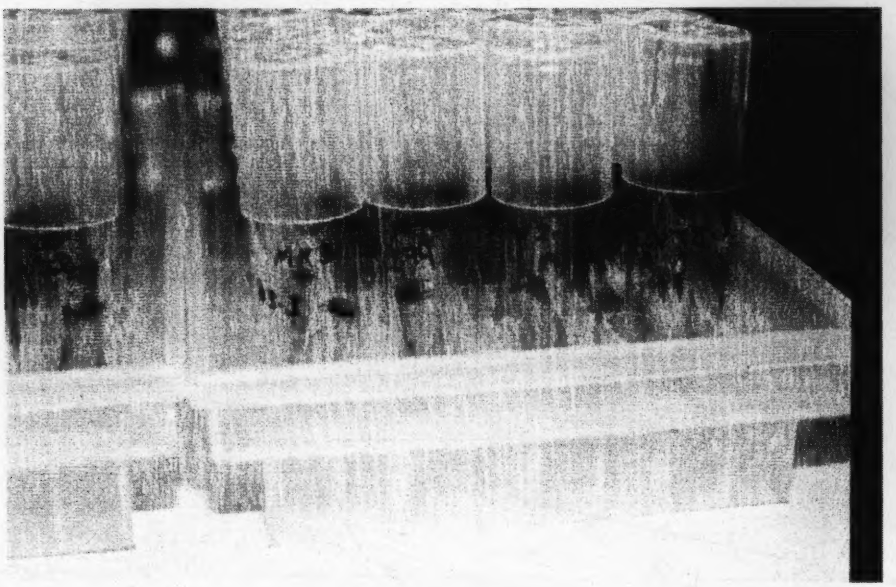

Figure 2-5. Tomato stem piece bioassay 1 week after inoculation.

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Chapter 3

Development of *Trichoderma harzianum* as a biological control for *Botrytis cinerea*[1].

3.1 Introduction

Control of plant disease is primarily focused on protecting plants from plant pathogens rather than curing plants after they have become diseased (Agrios 1997). The methods of disease control fall under 5 general categories: regulatory, cultural, biological, physical, and chemical (Agrios 1997). Regulatory control measures attempt to exclude a pathogen from a host or a geographic area (Agrios 1997). Cultural control measures are directed at preventing the pathogen from contacting the host under conditions favourable for disease development (Agrios 1997; Conway 1996). Biological controls aim to improve host plant resistance or favour organisms antagonistic to the pathogen. Physical and chemical control methods attempt to protect plants from inoculum that has, or is about to arrive (Agrios 1997).

Agricultural production practices are global issues and government policy and public opinion can dictate the production practices that are used (Conway 1996). Current concerns about reducing agricultural chemical inputs, as a means to improve the long-term sustainability of agriculture, favour the use of biological control as a method to reduce pesticide use (Conway 1996). Plant disease control in practice is often integrated, and consideration is given to all available options for disease control. For example, chemical controls will still be considered for disease control even when resistant varieties are grown. To be able to consider the use of a variety of disease control options requires that a variety of control options are available. This implies that the development of new control options is an important component of long-term success in disease control. Increased use of biological controls will only be realized when effective biological control options are available.

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Integrated management of plant diseases is proposed as an approach to disease control to reduce inputs into crop production by moving away from a sole reliance on fungicides (Conway 1996). The implication of this concept is that biological control agents are not expected to perform as well as fungicides, hence the requirement for integrating control measures to obtain satisfactory control (Harman 2000). The concern about the use of fungicides in disease control has been around since the 1970s (Agrios 1997). Increased emphasis on cultural control, resistant varieties and monitoring for the onset of disease development has formed the basis of integrated disease management which were primarily focused on reducing fungicide use (Agrios 1997).

Expectations that sustainable disease control can be obtained by integrated approaches that include biological control as a replacement for fungicides necessitates biological control performance equal to fungicides. Current integrated approaches to disease control still primarily involve fungicides to obtain satisfactory control (Agrios 1997). Improvements in cultural management and resistant varieties will still play important supporting roles but will not likely improve to the point where they will even collectively form the dominant component of disease control.

The knowledge that microorganisms have activity against plant pathogens has been recognized since the late 1920s, yet the commercial use of biological control is still rare (Andrews 1990, Agrios 1997). The lack of success with biological control is evidence that the interaction of biological control agents and pathogens in the agricultural environment is complex and there is much to learn about the selection and use of biological control agents in the field (Andrews 1990; Andrews 1992; Elad *et al.* 1996; Jacobsen and Backman 1993; Paulitz and Bélanger 2001).

The use of microorganisms forms the foundation of current approaches to biological control of plant disease. A number of different bacteria, yeasts, and fungi have been evaluated for use as biological control agents for a variety of diseases (Punja 1997). The mechanisms of action of biological control microorganisms are grouped into 3 broad categories; competition, antibiosis and mycoparasitism (Harman 2000). However, there is almost no understanding of how biological control actually operates in the field (Andrews 1990). A better understanding of how biological control operates in the complex field environment will improve the process and development of biological control agents (Andrews 1990, Elad and Chet 1995; Jacobsen and Backman 1993). In the interim, a practical approach to the development of effective, successful biological control agents is to assess potential agents in the field environment in which they are expected to perform (Andrews 1990; Andrews 1992).

Botrytis cinerea is an important pathogen of greenhouse tomato (*Lycopersicon esculentum* Mill.) and is able to infect, and cause disease on all aerial plant tissues (Howard *et al.* 1994; Utkhede *et al.* 2001). The most severe form of this disease is botrytis stem canker as it can result in the death of the plant and considerable economic loss (Calpas 2003; Utkhede *et al.* 2001). *Botrytis cinerea* epidemics can develop quickly under conducive environmental conditions. Control of *B. cinerea* in greenhouse crops is considered fairly straightforward in theory. The environmental conditions for disease development are understood and greenhouses offer the opportunity to modify the environment to prevent conditions favourable to disease development (Hausbeck and Moorman 1996).

Greenhouse environmental control is used to optimize crop growth and production (Calpas 2002). The dense crop canopy allows for the development of microclimates that favor the development of *B. cinerea*. It can be very difficult and cost prohibitive to use environmental control and energy inputs to maintain an environment that will prevent disease development and still optimize crop production (Menzies and Bélanger 1996; Paulitz and Bélanger 2001). Environmental modification for control of *B. cinerea* is certainly employed, however, other control options are often necessary (Calpas 2003).

A number of microorganisms have been studied for their biological control potential against *B. cinerea* including yeasts, bacteria and fungi (Dik *et al.* 1999; Elad *et al.* 1996). However, Trichodex®, a formulation of *T. harzianum*, is the only commercial product available for the biological control of *B. cinerea* (Dik *et al.* 1999; Bélanger and Paulitz 2001). Other species of *Trichoderma* spp. have demonstrated biologicial control potential against *B. cinerea*, including *T. viride* and *T. virens* (Dik *et al.* 1999). There is interest in the commercialization and registration of other strains of *T. harzianum* for the biological control of *B. cinerea* in crops including greenhouse tomato (Harman 2000; Ukthede *et al.* 2001; Utkhede and Mathur 2002).

Trichoderma harzianum strain (T-39) isolated from a cucumber fruit is the active agent in the biological control product Trichodex® (Elad *et al.* 1993). There is no information if there was a preliminary selection process that was used to identify the biological control potential of *T. harzianum* strain T-39 from among a number of strains evaluated against *B. cinerea*. Strain T-39 has been evaluated extensively against *B. cinerea* in greenhouse trials (Shtienberg and Elad 1997; Dik and Elad 1999). Strain T-39 has been demonstrated to reduce disease incidence of grey mould and the number of dead tomato plants caused by botrytis stem canker. (Dik and Elad 1999; Elad *et al.* 1995).

The performance of Trichodex® has generally been promising, however, inconsistent and variable performance is common (Moyano *et al.* 2003). Strategies have been devised to integrate the use of *T. harzianum* strain T-39 with fungicides in order to obtain satisfactory control of *B. cinerea* in commercial greenhouses (Dik and Elad 1999; Elad *et al.* 1995; Shteinberg and Elad 1997). It is possible that strain T-39, although a reasonably effective strain, is not the best strain for use against *B. cinerea* in greenhouse vegetable crops.

Recent studies evaluating the biological control activity of *T. harzianum* strain T-22, the active agent in Rootshield® have demonstrated that strain T-22 has potential to reduce stem canker lesion length, and plant death, caused by *B. cinerea* (Ukthede *et al.* 2001; Utkhede and Mathur 2002). The Rootshield® strain T-22 was a result of a protoplast fusion of two *T. harzianum* strains selected for improved rhizosphere competence and control of root diseases in turf (Harman 2000).

In vitro studies of antagonism often form the basis of preliminary studies assessing biological control potential of microorganisms (Edwards and Seddon 2001). Dual culture studies have been used to evaluating biological control activity of *Trichoderma* spp. against fungal pathogens (Bell *et al.* 1982; Dennis and Webster 1971) and have also been used to evaluate the effect of antagonists against *B. cinerea* (Moline *et al.* 1999; Edwards and Seddon 2001). Although individual strains of *Trichoderma* may demonstrate potential against a variety of pathogens it may be wise to evaluate biological control agents against individual plant pathogens in specific applications (Bell *et al.* 1982). The results of *in vitro* studies should be considered carefully, as although they may serve to reflect the capacity for antagonism but may not reflect the degree of biological control in the field (Bell *et al.* 1982).

The development of stem cankers is the most devastating phase of *B. cinerea* infection as stem cankers that girdle the stem kills all the tissue above the canker (Dik *et al.* 1999; Utkhede and Mathur 2002). Cankers on the main stem can effectively kill the plant resulting in the total loss of future yield potential of that plant. The ability to reduce the progression of stem cankers caused by *B. cinerea* represents a suitable criterion for evaluating the potential of biological control agents (Dik *et al.* 1999).

Tomato stem piece bioassays have been used to evaluate the biological control potential of antagonists, including *Trichoderma* spp. against *B. cinerea.*(O'Neill *et al.* 1996; Dik *et al.* 1999). However it was determined that wounds on live, whole plants remain susceptible to *B. cinerea* much longer than on detached stem pieces (Dik *et al.* 1999). Stem piece bioassays were used as the basis for preliminary selection of biological control agents prior to further evaluation of the most promising candidates in whole plant tomato petiole bioassays in the greenhouse (Dik *et al.* 1999).

Chemical control is currently the only effective method for controlling greenhouse epidemics of *B. cinerea*. The fungicides registered for control of *B. cinerea* in greenhouse tomato in Canada are; Rovral® (iprodione, Bayer), Ferbam® (ferric dimethyidithiocarbamate, UCB Chemicals) Exotherm Termil® (chlorothalonil, Rigo Company) and a Benlate ® (benomyl, Du Pont)–Manzate® (mancozeb, Du Pont) mix. The Benlate®-Manzate® combination is not registered for use on hydroponic greenhouse tomato crops. Currently there are no biological controls registered for the control of *B. cinerea* in Canada.

The objective of this study was;

1. Using the greenhouse tomato model system (Appendix 1.), develop a biological control for *B. cinerea* using Alberta strains of *Trichoderma* spp. and the criterion that the biological control agent must perform as well as fungicides.

Based on the information present in the literature, this is the first extensive study undertaken to collect and screen a large number of strains of *Trichoderma* spp. for the specific purpose of developing a biological control for *B. cinerea* in greenhouse tomato.

3.2 Materials and methods

3.2.1 Isolation and identification of strains of *Trichoderma* spp.

One hundred and eight strains of *Trichoderma* spp. were collected from various plant, soil, and compost sources in Alberta as well as from a culture collection (Dr. Ron Howard, Crop Diversification Centre South). The Rootshield® strain T-22, was included as the one hundred and ninth strain in this study. Recovery of strains from soil and compost samples was accomplished similar to the method used by Park *et al.* (1992). One-gram soil samples were added to 90 mL of sterile distilled water and homogenized for 1 minute, 1 mL of 10^4-10^5 serial dilutions of the suspensions were plated on *Trichoderma* spp. selective media similar to Elad *et al.* 1981 with the exception that streptomycin sulfate also was added. The media contained streptomycin sulfate (50 mg L^{-1}), chloramphenicol (3.0 g L^{-1}) and rose bengal (0.2 g L^{-1}) to supress the growth of bacteria. Recovery of *Trichoderma* spp. from plant sources was accomplished by recovering strains from actively growing colonies.

Strains were established and maintained in pure culture on malt extract agar (Difco®) using single conidia. Cultures were maintained on approximately 20 mL of malt extract agar in 8.5 cm diameter Petri plates at 20 °C under approximately 9 hr of natural light per day.

The first attempt to identify the strains of *Trichoderma* spp. was made using the key developed by Rifai (1969), the species determinations were then reassessed according to the new key developed by Gams and Bissett (1998). The species determinations of some of the strains were again evaluated based on the descriptions of new species of *Trichoderma* reported from Asia (Bissett *et al.* 2003). Reference cultures, selected by Dr. John Bissett, were obtained from the Canadian Collection of Fungal Cultures, Agriculture and Agri-Food Canada (Eastern Cereal and Oilseed Research Centre, Agriculture Canada, Ottawa, Ontario) and were used to aid the species determinations. Determinations were confirmed by Dr. John Bissett working with representative cultures of the individual species collected.

Species determinations were based on colony morphology and microscopic descriptions. Conidiophore structure and morphology were described from conidiophores taken from the edges of conidiogenous pustules or fascicles when conidia began to mature, usually after 4-7 days in culture.

3.2.2 Evaluation of the biological control potential of the strains of *Trichoderma* spp. for *Botrytis cinerea*

3.2.2.1 Preliminary subjective assessment of *Trichoderma* spp. antagonism against *Botrytis cinerea in vitro*

A preliminary characterization of antagonism between 102 strains of *Trichoderma* spp. and a single strain of *B. cinerea* (strain BN3) was carried out using a co-culture experiment on potato dextrose agar (PDA, BBL®). This study was undertaken before all the *Trichoderma* spp. had been collected and identified and before the final target *B. cinerea* strain had been selected (Chapter 2). *Botrytis cinerea* strain BN3 was selected for use in this study because it was collected from an Alberta tomato greenhouse experiencing severe botrytis blight.

A co-culture assessment, similar to that reported by Bell *et al.* (1982), was used. A single 5 mm agar (malt extract agar) plug was taken from a 2-week-old culture of each strain of *Trichoderma* spp. The agar plug was placed 10 mm from the edge of 8.5 cm diameter Petri plates containing approximately 20 mL of PDA. A 5 mm agar plug of a 2-week-old *B. cinerea* (strain BN3) was then placed 10 mm from the edge of the plate directly opposite the agar plug of the strain of *Trichoderma* sp. Two replicates of each strain of *Trichoderma* sp. - *B. cinerea* (strain BN3) pairing were established. The Petri plates were maintained at 20°C under approximately 9 hr of natural light per day.

Assessments were made at 1 and 2 weeks after the start of the trial. The activity of each *Trichoderma* spp. against *B. cinerea* was visually assessed and characterized as aggressive, moderately aggressive or weak based on the interactions in both replicate treatments.

Strains of *Trichoderma* spp. were considered aggressive if they began to grow over *B. cinerea* in 1 week, covered the entire Petri plate within 2 weeks, and sporulated where they grew over *B. cinerea*. *Trichoderma* strains were considered moderately aggressive if they were near, but not growing over, *B. cinerea* in 1 week, growing within the interaction zone or over a portion of the *B. cinerea* colony within 2 weeks, with sporulation evident where they grew over *B. cinerea*. *Trichoderma* spp. were considered weak if *B. cinerea* and *Trichoderma* exhibited distinct and separate regions of growth on the PDA medium after 1 week, with a clear zone of inhibition separating the 2 species. No growth of *Trichoderma* sp. over *B. cinerea* was observed after 2 weeks.

3.2.2.2 Effect of *Trichoderma* spp. on the radial growth of *Botrytis cinerea in vitro*

A second co-culture trial was undertaken to assess the biological control potential of the *Trichoderma* spp. strains against *B. cinerea*. Reduced radial growth of *B. cinerea* strain LF5 was measured as an indicator of growth inhibition by the *Trichoderma* spp. strains (Bell *et al.* 1982; Hermosa *et al.* 2000). *Botrytis cinerea* strain LF5 was selected as the target strain due to its virulence against tomato as determined in the tomato stem bioassay (Chapter 2).

The co-culture pairings were established on PDA and maintained as described above with the exception that *B. cinerea* strain LF5 was used instead of strain BN3. Four replicate treatments of each *Trichoderma* sp. - *B. cinerea* (strain LF5) pairing were assessed. The measurements were taken after 1 week of co-culture.

3.2.2.3 Evaluation of the effect of *Trichoderma* spp. on the development of *Botrytis cinerea* stem canker *in vivo* using a greenhouse tomato petiole bioassay

3.2.2.3.1 Evaluation of the effect of *Botrytis cinerea* spore suspension concentration on disease development using a greenhouse tomato plant petiole bioassay

To ensure consistent disease development on greenhouse tomato plants using a convenient method of inoculation, the target *B. cinerea* strain LF5 was evaluated for virulence against greenhouse tomato plants using the petiole bioassay.

Botrytis cinerea spore suspensions were made from 3-week old cultures that had been exposed to 14 hours of UV light (Philips 40 W lamp, F40T12/BLB) for the first 3 days of culture. The culture plates were maintained on PDA at 20°C under approximately 9 hr of natural light per day. *Botrytis cinerea* covered the plate in approximately 1 week with mature conidia covering the entire plate at 3 weeks. Later, at 3 weeks the culture plates were flooded with 5 mL of sterile distilled water with 1% Tween 80. A sterile wire loop was used to dislodge the spores from the conidiophores and the resulting suspension was extracted from the plate using a 1000 µL pipette. The spore suspension concentration was determined using a hemacytometer and adjusted to obtain the desired spore concentrations using sterile distilled water.

Spore suspensions were used as they represented a convenient and standardized method of inoculating the surfaces of tomato petioles. A droplet was easier to apply to a vertical surface than an agar disk on live plants in the greenhouse environment. The leaves of a growing crop are positioned horizontally and the cut surfaces of the petioles are then orientated vertically. Spore suspensions also provide a method to more easily standardize inoculation levels than do agar disks containing *B. cinerea*.

Three-month old greenhouse tomato plants (cv. Quest, De Ruiter Seed) were grown as described in a (120 m^2) polycarbonate research greenhouse at the Crop Diversification Centre South at Brooks, Alberta. The cultivar Quest was used because it was a cultivar grown by commercial greenhouse tomato growers. Tomato petioles were cut and inoculated with 10 μL droplets of *B. cinerea* spore suspensions at concentrations of 0, 10^3, 10^4, 10^5, 10^6, or 10^7 spores mL^{-1}. Eight successive petioles per plant were used, starting with the petiole on the first fully expanded leaf.

The petioles used in the bioassay were wounded by cutting the petiole perpendicular to the main axis of the petiole, approximately 10 cm from the tip of the leaf. This method of wounding was similar to Utkhede *et al.* (2001) and simulated the type of leaf damage commonly associated with workers moving through commercial tomato crop. Petiole and stem wounds are prime infection sites for *B. cinerea* under conditions of optimum disease development (Eden *et al.* 1996). Four replicate treatments arranged in a randomized design were evaluated for each spore concentration. The inoculated petioles were enclosed in a plastic bag immediately after inoculation in order to maintain conditions of high relative humidity around the wound site. This was similar to Eden *et al.* (1996) except that they used modified clear, lidded plastic pots containing salt solutions to maintain specific humidity levels around stem wounds. The length of petiole colonized by *B. cinerea* was measured 6 days after inoculation.

3.2.2.3.2 Observations of inoculum concentration resulting from a single sporulating stem canker caused by *Botrytis cinerea*

During the course of this study an opportunity arose to observe the inoculum concentration resulting from 1 heavily sporulating *B. cinerea* stem canker lesion in a tomato production greenhouse. The observations were made in a 120 m^2 tomato production greenhouse at the Crop Diversification Centre South at Brooks, Alberta. The tomato crop (cv. Trust, De Ruiter Seeds, cv. Quest, De Ruiter Seeds, and cv. Rapsodie, Novartis Seeds Inc.) was being grown to commercial standards according to Calpas (2003). A naturally occurring *B. cinerea* stem infection was found in the greenhouse and subsequent examination of all the plants in the greenhouse revealed that only 1 stem infection, representing a single locus, was present in the entire crop. There was also no evidence of any other *B. cinerea* infections of aerial plant tissues in the crop.

The stem canker had developed at a point on the stem where fertilizer feed solution was dripping onto the stem from a slow leak in an irrigation line. The resulting canker was measured and determined to be 1.5 cm wide by 14.5 cm long with an area of 21.8 cm^2. The presence of abundant sporulation was noted throughout the entire area of the lesion. The number of conidia cm^{-2} present on the sporulating lesion was estimated by determining the number of conidia cm^{-2} produced on heavily sporulating *B. cinerea* cultures (strain LF5) on 8.5 cm diameter Petri plates.

Six replicate culture plates (3-week old cultures) were flooded with 5 mL of sterile distilled water containing 0.1% Tween 80. A sterile wire loop was used to dislodge the spores and 1 mL of suspension was recovered from the plate. The spore concentration in the resulting suspension was determined using a hemacytometer. An average of the number of spores in the 6 suspensions was used to calculate the average number of spores cm^{-2} present on the sporulating culture plates.

Observations of conidial inoculum density were taken by collecting the terminal leaflet of the first, fully expanded, uppermost leaves from 36 of the 360 tomato plants throughout the greenhouse. Leaflets were collected from every fourth plant following transects along and across the plant rows (Figure 3-1). The rationale for collecting the uppermost, most fully expanded leaves was that these leaves were fully exposed to airborne spores and were not hidden or sheltered in the crop canopy.

The upper side of the terminal leaflet was gently pressed into the surface of a water agar Petri plate. The leaflet was then removed leaving a clear imprint of the leaflet in the water agar similar to the method described by Wickremasinghe *et al.* (1985). The area of the leaf imprint was divided into 20 (1 cm^2) sections by drawing lines on the bottom of the Petri plates. Spore counts were taken by examining the surface of the water agar plate under the microscope. Counts from an area of 0.009 mm^2 were taken from the center of each 1 cm^2 section of the leaflet imprint and were averaged to obtain an estimation of inoculum density of *B. cinerea*.

3.2.2.3.3 Effect of plant and petiole position on disease development

The tomato petiole bioassay was based on using more than 1 petiole per plant with the decision to use 8 petioles per plant based on the limited number of plants available and the magnitude of the assays contemplated. The assay was dependent on consistent disease reaction with *B. cinerea* across all petioles. It was important to determine if the disease reaction was dependent on petiole position. This experiment was undertaken to determine the effect of petiole position on the progression of *B. cinerea* in tomato petioles.

A spore suspension of *B. cinerea* strain LF5 was prepared and adjusted to 10^6 spores mL^{-1} as described previously. Four-month old tomato plants (cv. Quest, De Ruiter Seeds) were used in the study. Eight petioles per plant were selected for use starting with the petiole of the first fully expanded leaf as position 1. Each successive petiole up to the eighth petiole was used. The petioles were wounded as described previously and inoculated with 10 μL droplet of *B. cinerea* spore suspension. Four plants were used providing 4 replicates of each petiole position. The inoculated petioles were enclosed in a plastic bag immediately after inoculation in order to maintain conditions of high relative humidity around the wound site. The length of petiole colonized by *B. cinerea* was measured 6 days after inoculation.

3.2.2.3.4 Evaluation of *Trichoderma. harzianum* spore suspension concentration on *Botrytis cinerea* disease development using a greenhouse tomato petiole bioassay

In order to proceed to greenhouse screening trials to determine the biological control potential of the strains of *Trichoderma* spp., a trial was established to determine the optimum *T. harzianum* spore suspension concentration for use in screening trials. *Trichoderma harzianum* (*Trichoderma* strain 7) was used for evaluation against *B. cinerea* strain LF5.

Botrytis cinerea (strain LF5) spore suspensions were made by flooding 2- to 3-week-old cultures (PDA) with 5 mL of sterile distilled water containing 0.1% Tween 80. A sterile wire loop was used to dislodge the spores and 1 mL of suspension was recovered from the plate. The spore concentrations were adjusted to 10^6 spores mL^{-1} with sterile distilled water with the spore concentrations determined using a hemacytometer.

A greenhouse tomato plant petiole bioassay was conducted to determine the biological control potential of the strains of *Trichoderma* spp. This assay was conducted in a 120 m^2 polycarbonate greenhouse in a tomato crop grown at the Crop Diversification Centre South at Brooks, Alberta. Four-month old tomato plants (cv. Quest, De Ruiter Seeds) were used in the study. Eight petioles per plant were used, starting with the petiole on the first fully expanded leaf. The petioles used in the bioassay were wounded as described previously.

The cut surfaces were inoculated with a 10 µL droplet of a *B. cinerea* spore suspension at a concentration of 10^6 spores mL^{-1}. The petioles were then immediately inoculated with a 10 µL droplet of a spore suspension of *T. harzianum*. The *T. harzianum* spore suspension concentrations evaluated were 10^3, 10^4, 10^5, 10^6, and 10^7 spores mL^{-1}. There were 4 replicate treatments in a randomized design for each *T. harzianum* spore concentration. The petioles were enclosed in clear plastic bags immediately after inoculation in order to maintain conditions of high relative humidity around the wounds. The lesion lengths on petioles colonized by *B. cinerea* were measured one week after inoculation.

3.2.2.3.5 Greenhouse assessment of *Trichoderma* spp. biological control potential against *Botrytis cinerea* (strain LF5) using the tomato petiole bioassay

The biological control potential of the *Trichoderma* spp. strains was assessed using the greenhouse bioassay described previously. Five month–old tomato plants (cv. Quest, De Ruiter Seeds) were used in this study. Cut petiole surfaces were promptly inoculated with a 10 µL droplet of a *B. cinerea* spore suspension at a concentration of 10^6 spores mL^{-1}. The petioles were then immediately inoculated with a 10 µL droplet of a spore suspension (10^7 spores mL^{-1}) of *Trichoderma* spp. The petioles were enclosed in clear plastic bags after the dual inoculation in order to maintain conditions of high relative humidity around the wound. Eight replicates of each *Trichoderma* spp. strain treatments were assessed using this bioassay. Each experiment was assessed for the length of lesions caused by *B. cinere*a on petioles after 1 week. The lesion lengths were determined by measuring the brown discoloration of the petiole often accompanied by spore production. Due to the magnitude of the trials, the strains of *Trichoderma* spp. were assessed in separate experimental groups of 20 strains. The assay was conducted twice for each of the strains of *Trichoderma* spp. Seven promising strains were selected for a further study, and were evaluated for selection of the top 2 preforming strains. These 7 strains were 7, 18, 29, 93, 99, 105 and 108. All were strains of *T. harzianum* with the exception of strain 18, which was *T. virens*. These strains preformed well in both petiole bioassyas, except for strain 18 which preformed will in the first assay and not as well in the second.

3.2.3 Effectiveness of spray versus droplet application of *Trichoderma harzianum* for the biological control of *Botrytis cinerea* (LF5)

Spray applications of control agents are commonly used in the greenhouse environment. This trial was conducted to determine if effective biological control activity could be achieved from a spray application of *Trichoderma* sp. The top 2 *Trichoderma* sp. strains, 7 and 29, were evaluated using the greenhouse tomato petiole bioassay and comparing the spray versus droplet methods of delivery of *Trichoderma* sp.

Cut tomato petioles (cv. Quest, 5-month old plants) were first inoculated with a 10 μL droplet of *B. cinerea* (LF5) at 10^6 spores mL^{-1} and followed with inoculation of *Trichoderma* sp. via droplet or spray application. Two *T. harzianum* strains (7 and 29) were evaluated by application of a10 μL droplet of a suspension at 10^7 spores mL^{-1} or spray application of approximately 10 to 20 μL of the 10^7 spores mL^{-1} suspension. Eight replicate treatments of each *Trichoderma* sp. strain, and both spray and droplet application methods were used. The petioles were enclosed in clear plastic bags immediately after inoculation in order to maintain conditions of high relative humidity around the wounds. The tomato petioles were assessed after 1 week for the length of lesions caused by *B. cinerea*.

3.2.4 Evaluation of the effect of timing of spray application of the biological control activity of *Trichoderma harzianum* on *Botrytis cinerea* (LF5)

To expand on the effects of timing the application of the *Trichoderma* sp. on the biological control of *B. cinerea*, a second experiment was designed to simulate what could be expected to occur in a commercial greenhouse. *T. harzianum* (strains 7 and 29), were applied to cut greenhouse tomato petioles (cv. Quest, 5-month old plants) 4 replicate treatments, at 7 different timings of application. One treatment was inoculated with *T. harzianum* immediately after droplet application of *B. cinerea* LF5. The other treatments were inoculated with *T. harzianum* at 1, 2, and 3 hours before inoculation with *B. cinerea* strain LF5 or at 1, 2, and 3 hours after inoculation with *B. cinerea* strain LF5.

3.2.5 Greenhouse evaluation of *Trichoderma harzianum* biological control activity versus *Botrytis cinerea* compared to fungicides

Two trials were conducted to evaluate the performance of the top 2 *T. harzianum* biological control strains (strains 7 and 29) compared to fungicides that are registered for the control of botrytis blight in various crops including greenhouse tomato. The only exception was Quadris® (Syngenta), which does not have a registration for the control of *B. cinerea* but was included as a newer generation fungicide.

In the first trial, the *T. harzianum* strains were evaluated against Rovral® (iprodione 50%, Bayer) and a Benlate® (Benomyl 50%, DuPont)-Manzate® (Mancozeb 80%, DuPont) mix. The second trial was expanded to also include Captan® (Captan 50%, United Agri Products), Decree® (Fenhexamide 50%, Tomen Agro), and Quadris® (Azoxystrobin 250 g L^{-1}, Syngenta).

The trials were conducted using the greenhouse tomato petiole bioassay (cv. Quest, 5-month old plants) with 8 replicate treatments arranged in a randomized design. Each cut tomato petiole was first inoculated with 10 µL droplet of *B. cinerea* (LF5) at 10^6 spores mL^{-1} with the control agents applied immediately after by spray application. The fungicides were applied at the label rate; Rovral® at 1 g L^{-1}, Benlate® at 1.1 g L^{-1} plus Manzate ® (800 g kg^{-1}) at 2.25 kg ha^{-1}, Captan® at 6 g L^{-1}, Decree® at 1.7 kg ha^{-1}, and Quadris ® at 500 mL ha^{-1}.

Trichoderma harzianum strains 7 and 29 were evaluated by spray application of approximately 10 to 20 μL of a 10^7 spores mL^{-1} suspension. The petioles were enclosed in clear plastic bags immediately after inoculation in order to maintain conditions of high relative humidity around the wounds. The tomato petioles were assessed after 1 week by measuring the length of lesion on tomato petioles colonized by *B. cinerea*.

3.3 Statistical analyses

3.3.1 Effect of *Trichoderma* spp. on the radial growth of *Boytris cinerea in vitro*

These data were analyzed by an analysis of variance (ANOVA) using the general linear model (GLM) procedure (SAS Institute Inc.). The model used considered radial growth as a function of strain. Strain was treated as a random factor. The analysis was conducted using a 5% level of significance.

3.3.2 Effect of petiole position on disease development

The relationship between lesion length and petiole position was investigated as a repeated measures analysis using the Mixed procedure (SAS Institute Inc.). Akaike's information criterion was used to determine the appropriate covariance structure. The analysis was conducted using a 5% level of significance.

3.3.3 Greenhouse assessment of *Trichoderma* spp. biological control potential against *Botrytis cinerea* (strain LF5) using the tomato petiole bioassay.

These data were analyzed by an ANOVA using the GLM procedure (SAS Institute Inc.). The model used considered lesion length as a function of strain, date, and the interaction with petiole position as a covariate. The analyses were repeated excluding petiole position and considering lesion length as a function of strain, date and the strain by date interaction. All factors were treated as random factors. The analyses were conducted using a 5% level of significance.

The data from the third assessment of the final 7 *T. harzianum* strains were analyzed using an ANOVA using the GLM procedure. The model used considered lesion length as a function of strain, which was treated as a fixed factor. The treatment means were compared to the 5% level of significance using the Tukey test.

3.3.4 Effectiveness of spray versus droplet application of *Trichoderma harzianum* for the biological control of *Boytris cinerea* (LF5)

These data were analyzed using the Mixed procedure (SAS Institute Inc.). The Mixed procedure was used to model the heterogeneous variances among treatments. The model considered lesion length as a function of spray application method, *T. harzianum* biological control agent and the interaction. The analysis was conducted using a 5% level of significance.

3.3.5 Evaluation of the effect of timing of spray application of the biological control activity of *Trichoderma harzianum* on *Botrytis cinerea* (LF5)

These data were also analyzed using the Mixed procedure (SAS Institute Inc.) to model the heterogeneous variances among treatments. The model considered lesion length as a function of the *T. harzianum* biological control agent, the timing of application and the interaction. The analysis was conducted using a 5% level of significance.

As there were seven equally spaced treatments for timing, trends analysis was used to determine if there were linear and quadratic effects of timing and if these interacted with agent. These analyses were conducted using a 5% level of significance.

3.3.6 Greenhouse evaluation of *Trichoderma harzianum* biological control activity versus *Botrytis cinerea* (LF5) as compared to fungicides

These data were analyzed by an ANOVA using the GLM procedure (SAS Institute Inc.). The model used considered lesion length as a function of control agent. Control agent was treated as a fixed factor. The treatment means were compared to the 5% level of significance using the Tukey test.

3.4 Results

3.4.1 Isolation and identification of strains of *Trichoderma* spp.

One hundred and eight strains of *Trichoderma* spp. were collected from a number of sources (Table 3-1). Sixty-six of the strains were identified as *T. harzianum* Rifai, 33 were *T. rossicum* Bissett, Kubicek and Szakacs., 7 were *T. virens* (J. Miller, Giddens and Foster) von Arx, Beih., and 2 were *T. viride* Pers.:Fr. Descriptions of representative strains growing on malt extract agar are presented following the format of Gams and Bissett (1998).

Trichoderma harzianum (strain 7).

Colonies growing rapidly, 8.5 cm in diameter (filling the entire Petri plate) in 3 days at 20 °C. Conidiation initially effuse producing tufts or pustules, green, fringed with sterile white mycelium with in 6 days. Reverse colourless. Odour indistinct. Conidiophores tending to be regularly verticilliate forming a pyramidal structure. Phialides ampulliform to lageniform usually 3-4 verticillate, occasionally paired, mostly 4.0-7.5 x 3.0-3.9 μm, terminal phialides 9.6-10 μm long. Conidia subglobose to obovoid mostly 2.6-3.0 x 2.5-3.0 μm in diameter, smooth walled, pale green in colour at maturity.

Trichoderma viride (strain 27).

Colonies growing rapidly, 7.7 cm in diameter in 3 days at 20 °C. Conidiation forming compact pustules; white at first, eventually green. Reverse colourless. Odour distinctly coconut. Conidiophores relatively narrow and flexuous; with primary branches, usually paired or in whorls of three, usually short and not extensively rebranched. Phialides solitary or in verticils of 2 or 3, lageniform, often curved, 7-12 x 2.4-3.0 μm . Conidia more or less roughened mostly 4.5-5.0 x 4.3-4.6 μm in diameter, green at maturity.

Trichoderma virens (strain 18).

Colonies growing rapidly, 8.5 cm in diameter (filling the entire Petri plate) in 3 days at 20 °C. Conidiation effuse, covering the entire surface of the plate, forming flat pustules concentrating near the margins of the plate; quickly turning dark bluish-green. Reverse colourless. Odour indistinct. Conidiophores in areas of effuse conidiation arising as lateral branches from undifferentiated mycelium, sterile at the base for about half the length, irregularly branched at the top half, terminating in a cluster of 3-6 closely appressed phialides. Macronematous conidiophores,branching irregularly, the upper part fertile to the apex and the apex bearing a terminal whorl of appressed branches and phialides. Phialides lageniform to ampulliform, 4.5-10 x 2.8-5.5 µm. Conidia broadly ellipsoidal to obovoid 3.0-5.2 x 2.4–3.0 µm, green to olive at maturity

Trichoderma rossicum (strain 82).

Colonies growing 6.7 cm in diameter in 3 days at 20°C. Reverse colourless to dull yellowish. Odour indistinct. Conidiation aerial mycelium usually lacking on malt extract agar, condiophores arising from hemispherical pustules developing after 7 days on malt extract agar. Conidiophores relatively broad and straight, primary branches usually paired, terminal phialide bearing branches short cylindrical. Phialides arising on the terminal branches usually in whorls of 2, or 2-4 vericillate on subterminal branches, ampulliform, mostly 4.5-7.0 x 3.0-5.4 µm, abruptly narrowed to a short collula. Conidia short cylindrical with rounded ends, 3.0-4.5 x 2.0-3.0 µm, hyaline to green at maturity.

The strains of *T. rossicum* collected in the course of this study represent the first Canadian records of this species.

Images of colony morphology of a representative colony of each of the 4 species on malt extract agar at 4 and 6 days of age are presented in Figures 3-2 and 3-3 respectively. Microscope images exhibiting characteristic conidiophore morphology from a representative strain of each of the 4 species are presented in Figures 3-4 and 3-5.

3.4.2 Evaluation of the biological control potential of the strains of *Trichoderma* spp. against *Botrytis cinerea*

3.4.2.1 *In vitro* co-culture assessments of the biological control potential of the strains of *Trichoderma* spp. against *Botrytis cinerea*

The *Trichoderma* spp. response to *B. cinerea* (strain BN3) in co-culture was grouped into 3 subjective categories; aggressive, moderately aggressive and weak (Table 3-2). The results of the second co-culture evaluation, determining the ability of the

Trichoderma spp. strains to reduce the radial growth of *B. cinerea* (strain LF5), are presented in Table 3-3. All of the strains of *Trichoderma* spp. reduced the radial growth of *B. cinerea* from 53% to 69%. Greater reductions in the radial growth of *B. cinerea* were interpreted as result of a more aggressive *Trichoderma* spp. response.

3.4.2.2 Evaluation of the effect of *Trichoderma* spp. on the development of *Botrytis cinerea* stem canker *in vivo* using the greenhouse tomato petiole bioassay

The mean lengths of tomato petiole colonized by *B. cinerea* strain LF5 over increasing levels of spore suspension concentrations is presented in Figure 3-6. This experiment was also repeated with similar results. Based on the results, a *B. cinerea*, strain LF5, spore suspension concentration of 10^6 spores mL^{-1} was selected for use in all subsequent tomato bioassay trials. The analysis of the effect of petiole position on lesion development assumed compound symmetry based on Akaike's information criterion. Analysis of petiole position on lesion development revealed a significant linear trend with decreasing lesion lengths as petiole position increased from 1 to 8 (Table 3-4). Lesion lengths became shorter as petiole age increased. The linear relationship is described by the equation: $y = 49.00 - 1.28 \times$ petiole.

The magnitude of greenhouse tomato petiole bioassay to assess biological control activity of *Trichoderma* spp.against *B. cinerea* (LF5) required that the biological control strains be assessed in groups of approximately 20 strains. The tomato petiole bioassay was conducted using spore suspensions of *Trichoderma* spp. to inoculate the tomato petioles. On the basis of the evaluation of the effect of *T. harzianum* (strain 7) spore suspension concentration on the length of tomato petiole colonized by *B. cinerea* (LF5) (Figure 3-7), *Trichoderma sp.* spore suspensions of 10^7 spores mL^{-1} were selected for use in the tomato petiole bioassay evaluations.

Observations of the natural inoculum density as determined by the leaf print assay is presented in Table 3-5. The number of *B. cinerea* conidia on the leaflets ranged from 5.0×10^2 conidia cm^{-2} to 8.4×10^4 conidia cm^{-2} resulting from 1 sporulating lesion of an area of 21.8 cm^2. The sporulating culture plate assessment resulted in an average of 1.49 x 10^8 conidia per culture plate, equaling 1.1 x 10^7 conidia cm^{-2}. Based on this figure it was estimated that a total of 2.4 x 10^8 conidia were present on the sporulating stem lesion.

The results of the tomato petiole bioassays are presented in Tables 3-6, 3-7, 3-8, 3-9 and 3-10. The greenhouse bioassay more closely approximates a commercial greenhouse tomato crop, the actual environment that the strains of *Trichoderma* spp. would be expected to perform. The biological control ability of the strains of *Trichoderma* spp. was assessed based on their ability to reduce the length of petiole colonized by *B. cinerea* as compared to the negative control, where the petiole was only inoculated with *B. cinerea*. Examples of negative and positive control petiole responses are presented in Figures 3-8 and 3-9 respectively.

The tomato petiole bioassy assessment of *Trichoderma* spp. biological control activity against *B. cinerea* (LF5) was repeated once with similar results. The preformance of strains of *Trichoderma* spp in reducing the length of tomato petiole colonized by *B. cinerea* ranged from 22% to 97% in the first evaluation and 19% to 89% in the second evaluation.

The analysis of the tomato petiole bioassay data first included petiole position as a factor. The results of the covariance analysis demonstrated that petiole position and all interactions between date, strain and petiole position were not significant. Petiole position was then removed from the analysis.

The performance of strains of *Trichoderma* spp. in reducing the lengths of lesions on tomato petioles colonized by *B. cinerea* over both trial dates ranged from 6% to 87%. The Rootshield® strain, T-22, reduced petiole lesion lengths by 56%.

The ANOVAs of the tomato petiole bioassay data demonstrate that the significance of the main effects of strain, date and the strain by date interaction varied with each of the 5 groups of strains assessed. The results from the analysis of the performance of strains 1 to 18 (Table 3-6) demonstrates that the differences in lesion length due to strain was not significant. Trial date and the strain by date interaction were also not significant.

The results from the analysis of the performance of strains 19 to 48 (Table 3-7) demonstrate that strain, date and the interaction all had a significant effect on lesion length. The differences among the strains were significant, the differences between the 2 trial dates was significant and the significant effect of the interaction demonstrates that the relative performance and ranking of the strains was not the same on both trial dates.

The analysis of the data from strains 49 to 68 (Table 3-8) revealed only a significant effect of date, as did the analysis of the data from strains 69 to 88 (Table 3-9). The analysis of the data from strains 89 to 109 (Table 3-10) did not reveal any significant effect of strain, date or the interaction.

3.4.3 Selection of the most promising biological control strains of *Trichoderma* spp.

All assays to characterize the biological control potential of the *Trichoderma* spp. against *B. cinerea* differentiated the performance of the strains of *Trichoderma* spp. It was evident that a number of strains of *Trichoderma* spp. showed promise for development as potential biological control agents however a selection was made to narrow the field to some of the most promising candidate strains for further studies.

Two *T. harzianum* biological control strains (7 and 29) were selected on their overall performance in both sets of co-culture experiments (Tables 3-2 and 3-3) and the tomato petiole bioassays (Tables 3-6, 3-7, 3-8, 3-9, 3-10 and 3-11). Greater emphasis was given to *Trichoderma* spp. performance in the greenhouse tomato petiole bioassays. These 2 strains were 7 and 29, and were both strains of *T. harzianum*. Strain 29 performed well in all assays. Strain 7 was characterized as weak in the first co-culture experiment but performed well in the second co-culture experiment and the tomato petiole bioassays. Strains 7 and 29 reduced the radial growth of *B. cinerea* by 61 and 69%, respectively, in the second co-culture assessment. They also reduced the length of tomato petiole colonized by *B. cinerea* by 79% and 87%, respectively, in the tomato petiole bioassay. The Rootsheild® strain, T-22, performed well in the second co-culture experiment but was not outstanding in the petiole bioassay.

The analysis of the 7 selected *T. harzianum* strains (Table 3-11) demonstrated that strains 7 and 29 were the top performing strains, however only strain 29 was significantly different from the *B. cinerea* positive control.Strains 7 and 29 reduced the length of tomato petiole colonized by *B. cinerea* by 56 and 77%, respectively, when compared to the inoculated control in this third study.

The results of the comparison of the effect of droplet versus spray application on the efficacy of the *T. harzianum* strains 7 and 29 using the tomato petiole bioassay are presented in Table 3-12. The analyses of these data demonstrated no significant difference between spray or droplet application method on the performance of the biological control strains 7 and 29. The 2 biological control strains were not significantly different, however both strains were significantly different from the *B. cinerea* positive control. A 95% confidence interval calculated on the difference in lesion lengths caused by the *B. cinerea* positive control and the biological control strains was 31.97 ± 9.32 mm.

T. harzianum strains 7 and 29 are equally effective in controlling *B. cinerea* when applied up to 3 hours before or 3 hours after inoculations with *B. cinerea*. The results of this study are presented in Table 3-13. Trends analysis of these data demonstrated that there were no linear and quadratic effects of timing of application of the biological control strains 7 and 29 on the performance of these strains. In addition there was no interaction of timing of interaction and strain. Simple comparisons of the performance of the biological control strains demonstrated that strains 7 and 29 were not significantly different from one another, but both provided significant control of *B. cinerea*.

The results of both trials comparing the relative performance of *T. harzianum* strains 7 and 29 to fungicides in controlling *B. cinerea* are presented in Tables 3-14 and 3-15. There are differences in the control of *B. cinerea* with iprodione providing the best level of control in the first comparison and *T. harzianum* (strain 29) providing best level of control in trial 2, but the differences between the chemical and biological control strains are not significant in either trial. This demonstrates that the *T. harzianum* biological control agents are as effective as the fungicides. Orthogonal comparisons of both trials showed that both the biological controls and fungicides provided significant ($P \leq 0.05$) control of *B. cinerea* and that there was no significant difference ($P \leq 0.05$) in control provided by the fungicides or *T. harzianum* control agents. The level of control provided by all control agents ranged from 64% to 94% reduction of tomato petiole colonized by *B. cinerea* in the first trial and 56% to 88% in the second trial.

Another factor considered in the selection process was that the strains sporulate abundantly in culture. Adequate spore production was considered as an important characteristic for commercial production and use of the biological control agent. Although sporulation was not studied, it was assessed visually when the selections of the top performing *Trichoderma* strains were made. Both *T. harzianum* strains 7 and 29 sporulated readily and abundantly in culture.

3.5 Discussion

3.5.1 Isolation and identification of the strains of *Trichoderma* spp.

Strains of *Trichoderma* spp. were readily isolated from soil and compost samples and accounted for the majority of the collected strains. *Trichoderma* spp. have a worldwide distribution and are frequently dominant components of soil microflora in a variety of habitats (Gams and Bissett 1998; Howell 2003).

Trichoderma spp. can be recognized by common macroscopic characters including rapid growth in culture with little aerial mycelium, and the production of distinctive greenish conidiogenous pustules (Bissett 1984, 1991). The genus is characterized by repeatedly branched conidiophores, flask shaped phialides with hyaline or green colored conidia (Bisset 1984, Gams and Bissett1998).

Persoon introduced the genus in 1794 where he described four species including *T. viride* (Bissett 1991; Rifai 1969). Rifai's (1969) work provided the basis for generic delimitation based on microscopic characters still generally accepted today. Rifai (1969) also recognized the great degree of diversity in the genus and introduced the concept of species aggregates to describe very similar, hardly separable species. The species aggregates apply the concept of species in the broadest sense (Gams and Myer 1998).

Rifai (1969) understood that his species aggregates were not biological species (Bissett *et al.* 2003) and that future work would likely narrow the specific delimitations. The hardly separable species may some day become separable.

Gams and Bissett (1998) present the most recent revision of the genus that expands the number of species well beyond the nine aggregates proposed by Rifai (1969). Gams and Bissett (1998) divided the genus into 3 sections collectively containing 34 species of *Trichoderma* including descriptions of anamorphic stages of *Hypocrea* Fr. and *Podostroma* P. Karst. The treatment by Gams and Bisset (1998) retained the species aggregate concept in reference to *T. harzianum* and *T. viride*. Seven new species of *Trichoderma* were introduced by Bissett *et al.* (2003).

Teleomorph associations in the genus *Hypocrea* have been known since 1895 however the use of teleomorph associations in the delimitation of *Trichoderma* is problematic. *Trichoderma* spp. are also known to have teleomorphs in the related genera *Podostroma* and *Sarawakus* Boedijn (Gams and Bisset 1998). In addition, species of *Hypocrea* have anamorphs that are not easily recognized as *Trichoderma* and have been described as *Gliocladium* Corda, *Verticillium* Nees or *Acremonium* Link ex. Fr. (Bissett 1991). To complicate the situation further, teleomorphs are not known for many of the *Trichoderma* anamorphs (Bissett 1991).

Final resolution of species in *Trichoderma* is expected to rely on the integration of molecular characters with morphological characters and life cycle associations with the teleomorphs (Bisset *et al.* 2003).

Trichoderma harzianum is one of the most common species within the genus and is commonly known as a biological control agent against a wide variety of plant pathogenic fungi (Gams and Meyer 1998). The majority of the strains collected in this study were *T. harzianum* strains with 66 of the 109 strains. The 33 *T. rossicum* strains collected in this study represent the first Canadian record of this species, the type culture of *T. rossicum* originating from Siberia, Russia and described by Bissett *et al.* (2003).

3.5.2 Evaluation of the biological control potential of the strains of *Trichoderma* spp. against *Botrytis cinerea*

In vitro co-culture assays have been used to evaluate biological control potential of strains of *Trichoderma* spp. against a number of plant pathogenic fungi. The assays have used subjective criteria as a measure of *Trichoderma* spp. antagonism against the target pathogens (Bell *et al.* 1982; Hermosa *et al.* 2000) and growth inhibition of the target pathogens (Dennis and Webster 1971). In some cases the investigation of antagonism due to the production of volatiles by *Trichoderma* spp. form the basis of the assay (Dennis and Webster 1971).

This study has differentiated the strains of *Trichoderma* spp based on antagonistic activities against specific plant pathogens as well as demonstrating that antagonism of specific strains of *Trichoderma* spp. vary across different pathogens. The results from these studies indicate the genetic diversity present in strains and that a number of mechanisms are involved in the antagonism by *Trichoderma* spp. *In vitro* assays are not expected to necessarily relate to the degree of biological control in the field but they are expected to reflect some capacity of the biological control potential (Bell *et al.* 1982).

Dual culture studies are useful, however drawing conclusions on biological control potential and mode of action based on these studies must be done carefully. Edwards and Seddon (2001) demonstrated that the mode of action of *Brevibacillus brevis* Nagano against *B. cinerea* in culture was not likely the same mechanism that was operating in the field. The use of *in vitro* screening studies could have the consequence of excluding promising biological control organisms from further study as well as including strains that will not perform as well in the field.

Both of the co-culture assays employed in this study were simple to conduct but were abstract in that they do not involve the host crop for which the biological control was being developed. However, the co-culture assays did identify both *T. harzianum* strains 7 and 29 as having biological control potential. The first co-culture assessment, the subjective assessment, did not identify strain 7 as having potential for further development, but the second, objective co-culture assay did identify biological control potential in strain 7. These results indicate the potential for excluding promising strains from further evaluation if single *in vitro* assessments were used exclusively in the initial screening of biological control candidates.

Andrews (1992) discusses that the screening process for potential biological control agents must be both efficient and a reliable indicator of field performance. *In vitro* screening studies provide an inexpensive means to screen large numbers of biological control candidates (Andrews 1992). Field assays are realistic, but may be logistically impractical, too expensive, and could be too severe, causing false negatives (Andrews 1992).

In the context of these statements it is important to realize that it is only when a biological control organism has been registered for use that the true field trials begin, in commercial agriculture. Failure to perform or inconsistent performance in the field is also very expensive both in direct losses incurred by producers and in terms of losses associated with potential poor returns on the research investment and cost of commercialization. Although co-culture assays are useful, the selection of any biological control agent should always include some assessment of the control agent in the actual crop and environment for which it is intended. The field assessment should be included in early in the mass screening process, and should be rigourous, involving a known, highly virulent strain of the pathogen.

Some studies evaluating potential microbial biological control agents for biological control of *B. cinerea* have used stem piece bioassays as a method of mass screening (Dik *et al.* 1999). While these assays may be more relevant to the field than culture plate studies, they are still an abstraction and not a substitute for a live plant in the crop production environment. It has been demonstrated in tomato that wounds on live plants remain susceptible to infection much longer than on wounds stem segments (O'Neill *et al.* 1996).

Assays that involve live plants in a high yielding crop production environment more closely simulate the actual commercial environment and improves the relevance and rigour of the assay. High yielding tomato plants in high density crop stands are under considerable stress to set and fill fruit (Calpas 2003) and may also be more susceptible to infection by *B. cinerea* than vegetative tomato plants under low density or low yielding situations.

The greenhouse tomato petiole bioassay provided the most direct assessment of biological control potential as it involved the biological control agent, the target pathogen and the host crop. It did not provide information for why some potential biological control agents perform better than others with respect to specific modes of action, but it was the most direct measure of how well the biological control organism can be expected to perform.

None of the assays used in this study was based on any specific indicators of the mode of action of *Trichoderma* spp., such as differential production of enzymes such as chitinases or glucanases. Even when using these specific indicators to select the most promising biological control strains, commercialization assessments would still have to include trials in the intended environment and the target host crop.

Of the three assays used to assess *Trichoderma* spp. biological control potential against *B. cinerea*, the greenhouse tomato petiole bioassay gave the widest discrimination in biological control performance between the strains of *Trichoderma* spp. The assay is particularly useful because it is focused on the performance of *Trichoderma* spp. strains against *B. cinerea* in the same situation for which the biological control was being developed. The greenhouse tomato petiole bioassay was also the most intensive assay as it required large facilities and capabilities to grow a high yielding greenhouse tomato crop.

The primary concern with the development of *B. cinerea* stem cankers is the death of the plants and the associated yield loss. The death of an individual tomato plant can represent a yield loss exceeding 25 kg/m^2 (Calpas 2003) and growers will go to considerable effort to prevent or control the development of *B. cinerea* stem cankers. Control methods are often integrated and growers will attempt to modify the environment to prevent optimum conditions for disease development and use fungicides to protect against infection.

Any action to limit direct wounding of the stem would reduce the likelihood of stem infections. Greenhouse tomato crops require routine harvesting and pruning that wound the plant. When wounding is an inevitable part of crop management, wound placement may have an effect on the likelihood of stem infection and canker development. Removal of leaves directly at the node creates wounds directly on the stem. A strategy employed by some growers is to prune to leave 3 centimeters of petiole stub still attached to the stem. This places the wound a distance away from the stem. If stem wounds are equally susceptible to infection by *B. cinerea* as petiole wounds, it is reasonable to expect that infection of a wound directly on the stem would result in a stem canker.

Verhoeff (1968) demonstrated that breaking off the petioles close to the stem resulted in the lowest incidence of stem lesions on tomato plants and reported that this method of leaf pruning was the standard for growers. Leaving 3 to 5 centimeters of petiole attached to the stem was considered to provide an excellent starting point for *B. cinerea* to colonize and subsequently invade the stem (Verhoeff 1968).

However, having the wound a few centimeters away from the stem provides the opportunity to intervene with petiole infections before they reach the stem and develop into stem cankers. Tomato plants are generally pruned twice a week and petiole infections can be identified and treated before the canker reaches the stem. Treatment primarily involves excising the infected portion of the petiole. As this treatment also involves the creation of a wound, an application of fungicide is often included under conditions of disease pressure. The tomato petiole bioassay simulates this situation where the biological controls can be evaluated and developed to protect the wound from colonization by *B. cinerea* and provide an acceptable alternative for fungicide use.

Other stem assays evaluating the efficacy of RootShield® (*T. harzianum* strain T-22), use the length stem lesion development, or the number of stem lesions over the course of the entire season as indicators of biological control efficacy (Dik and Elad 1999; Utkhede and Mathur 2002). In 1 study, the RootShield® treatment resulted in fewer dead plants than the *B. cinerea* inoculated control, however 17% of the plants still died due to *Botrytis* stem canker in the RootShield® treatment (Utkhede and Mathur 2002). This level of loss would be disastrous for a commercial greenhouse operation.

The tomato petiole bioassay allows for the integration of the assessment of biological control performance with the option of subsequent cultural control by excising infected petioles. This reflects what could be expected in ongoing disease control programs in some commercial greenhouses to limit the loss of entire plants to stem canker caused by *B. cinerea*. Infected petiole tissue was excised and the resulting wounds were monitored to ensure that any subsequent infections did not occur. The tomato crop was maintained to commercial standards at all times.

The early assessments with the greenhouse tomato petiole bioassay used droplet application to deliver the *Trichoderma* spp. spore suspensions to the cut surface of the petiole immediately after inoculation with *B. cinerea*. Droplet application is a convenient method for delivery of precise volumes of spore suspensions, however it is not a practical method for application of a biological control agent in commercial greenhouses. Spray applications are commonly used for the delivery of disease control agents in commercial tomato greenhouses. Spray application of spore suspensions was demonstrated to be an effective method of delivering the *T. harzianum* biological control strains.

Observations of the natural inoculum density resulting from 1 sporulating stem lesion of an area of 21.8 cm^2 with an estimated 2.4 x 10^8 spores. The sporulating culture plate assessment resulted in an average of 1.49 x 10^8 conidia per culture plate, equaling 1.1 x 10^7 conidia cm^{-2}. Weeds *et al.* (1999) reported recovering 2.2 x 10^8 conidia cm^{-2} from Petri plates containing 8-10 day old *B. cinerea* colonies on malt extract agar. Köhl *et al.* (1995) reported 3.7 x 10^5 conidia cm^{-2} of *Botrytis allii* on segments of dead onion leaves. Nicot *et al.* (1996) obtained 10^8 conidia from *B. cinerea* Petri plate cultures growing on PDA and 1.5 x 10^7 conidia from 2 cm long infected stem pieces. Based on these findings the estimated number of conidia present on the stem lesion is reasonable.

Conidial densities on the leaflets were in the range of 10^4 conidia cm^{-2}. The inoculum density was determined by the leaf print method, which has been demonstrated to be a consistent and efficient method to assess spore populations on leaves (Wickremansinge *et al.* 1985). No studies were conducted to determine the viability of the conidia. It was not known how long the sporulating stem lesion was present. The conidial densities on the leaflets must be considered as the cumulative result of spore production over some period of time.

Inoculation of the tomato petiole wounds with *B. cinerea* was accomplished by applying 10 µL of a 10^6 spore mL^{-1} conidial suspension, resulting in 10^4 spores actually applied. The surface area of the cut petiole surface was approximately 0.5 cm^2. The *B. cinerea* inoculum load applied in the stem piece bioassay was approximately twice that observed the natural conidial densities. Inoculation of tomato plant material with *B. cinerea* in similar studies has often been accomplished using 10^3-10^6 conidial suspensions applied by droplet, mist, or spray (Dik *et al.* 1999, Eden *et al.* 1996; O'Neill *et al.* 1996 Ukthede *et al.* 2001; Utkhede and Mathur 2002).

Trichoderma harzianum strains 7 and 29 are equally effective in controlling *B. cinerea* when applied to petiole wounds as individual treatments 1, 2 and 3 hours before the application of *B. cinerea* and as individual treatments 1, 2, and 3 hours after the application of *B. cinerea*. These results demonstrate that foliar application of *T. harzianum* is a practical approach for a commercial greenhouse operation where a spray application could be expected to contact wound sites, caused by routine plant maintenance, before or after actual infection by *B. cinerea*.

Although Utkhede *et al.* (2001) reported curative control of *B. cinerea* stem canker in greenhouse tomato using *T. harzianum*, some studies have concluded that *T. harzianum* can prevent infection of wounds when applied together with *B. cinerea* but will not affect the progress of the lesion once infection has established (O'Neill *et al.* 1996). Determining the point when an infection has established is challenging.

Infection of greenhouse plants by *B. cinerea* is likely to occur via conidia (Kerssies *et al.*1997) and conidia take approximately 6-10 hours to germinate after the onset of favourable conditions (Epton and Richmond 1980). A *B. cinerea* infection could be expected to establish in approximately 10 hours. Although the number of hours in the workday for an average tomato greenhouse can vary, it is reasonable to expect that all whole-crop pruning operations are completed within 1 day (Calpas 2003). This also makes it possible for a biological control application to be made the same day, likely before *B. cinerea* establishes new infections. The results of this experiment demonstrate that effective control of *B. cinerea* can be obtained by applying *T. harzianum* strains 7 and 29 up to 3 hours after inoculation by *B. cinerea*. *Trichoderma* spp. are known for rapid growth (Howell 2003) and this quality likely contributes to their competitive advantage in colonizing wounds.

No information was obtained on the mode of action of *Trichoderma* spp. strains evaluated in this study. Competition is reported to be the primary mode of action of Trichodex ® (*T. harzianum* strain T-39) against *B. cinerea* (Elad and Kapat 1999). Strain T-39 does not act through volatile or non-volatile antibiotics (Elad and Kapat 1999). No mycoparasitism has been observed in the interaction between *T. harzianum* strain T-39 and *B. cinerea* (Elad and Kapat 1999). However, protease activity is suggested as being involved in the biological control of *B. cinerea* by *T. harzianum* strain T-39 (Elad and Kapat 1999).

The activity of secondary compounds such as 6-pentyl-a-pyrone have been demonstrated to be involved in the activity of *T. harzianum* strains against some plant pathogenic fungi (Scarselletti and Faull 1994). Many enzymes including endo-ß-1,6-glucanases, chitinases, and proteases have been demonstrated in the antagonistic activity of *T. harzianum* (Deane *et al.* 1998; del la Cruz *et al.*1995; Harman *et al.* 1993). Enzymes of *T. harzianum* were shown to work synergistically against plant pathogenic fungi (Schirmböck *et al.* 1994).

The reaction of strains of *T. harzianum* and various plant pathogenic fungi vary and are based on different mechanisms (Harman 2000; Howell 2003). Many genes are involved in the antagonistic activities of *T. harzianum* (Cortes *et al.* 1998; Harman 2000, Mach *et al.* 1999). The high degree of variability in the inhibition of *B. cinerea* petiole colonization exhibited by the different strains of *T. harzianum* in the tomato petiole bioassay supports the likelihood of the involvement of many genes in this interaction.

3.6 Conclusions

The 2 most promising *T. harzianum* strains 7 and 29 were as effective as fungicides in controlling *B. cinerea* in greenhouse tomato. Both the biological control agent and fungicide treatments reduced the length of petiole colonization to a level that would allow an opportunity for greenhouse growers to excise the infected tissue and re-treat with a control agent in order to protect the subsequent wound from infection before the lesion progressed into the main stem.

The tomato petiole assay used in the selection of *T. harzianum* strains 7 and 29 as biologicial control agents integrates the use of the biological control agents with cultural methods to prevent petiole infections from reaching the main stem. This approach will require repeated application of the biological control strains in order to place *T. harzianum* in contact with *B. cinerea* on wound sites.

The classical approach to biological control includes the expectation that the biological control agent, once released, will maintain itself in the environment and provide ongoing control (Conway 1996). Industry may not be interested in developing products that are self perpetuating as there is an associated limited return on investment (Conway 1996). The repeated release of biological control organisms represents a second scenario for use and implies enhanced profitability of biological control agents. The long-term success of biological control of *B. cinerea* will be linked to effective control agents that are profitable to develop and commercialize.

The tomato petiole bioassay proved to be a good method to evaluate large numbers of *Trichoderma* spp. biological control candidates in a commercial greenhouse tomato production system. This study demonstrates that Alberta strains of, *T. harzianum* (strains 7 and 29) have excellent potential for commercialization as biological control agents for *B. cinerea* in greenhouse tomato. The *T. harzianum* strains 7 and 29 perform equally well as fungicides for the control of *B. cinerea*.

3.7 Tables and figures

Table 3-1. One hundred and nine strains of *Trichoderma* spp. included in this study.

Strain	**Species substrate**	**Collection**	**Collection Location Alberta, CANADA**
1	*T. harzianum*	Wood bark compost	Cochrane
2	*T. harzianum*	Wood bark compost	Cochrane
3	*T. harzianum*	Wood bark compost	Cochrane
4	*T. harzianum*	Wood bark compost	Cochrane
5	*T. harzianum*	Wood bark compost	Cochrane
6	*T. virens*	Wood bark compost	Cochrane
7	*T. harzianum*	Wood bark compost	Cochrane
8	*T. harzianum*	Wood bark compost	Cochrane
9	*T. virens*	Wood bark compost	Cochrane
10	*T. harzianum*	Wood bark compost	Cochrane
11	*T. harzianum*	Wood bark compost	Cochrane
12	*T. harzianum*	Wood bark compost	Cochrane
13	*T. harzianum*	Wood bark compost	Cochrane
14	*T. harzianum*	Wood bark compost	Cochrane
15	*T. harzianum*	Wood bark compost	Cochrane
16	*T. harzianum*	Wood bark compost	Cochrane
17	*T. virens*	Wood bark compost	Cochrane
18	*T. virens*	Wood bark compost	Cochrane
19	*T. virens*	Wood bark compost	Cochrane
20	*T. harzianum*	Wood bark compost	Cochrane
21	*T. harzianum*	Wood bark compost	Cochrane
22	*T. harzianum*	Wood bark compost	Cochrane
23	*T. harzianum*	Wood bark compost	Cochrane
24	*T. virens*	Wood bark compost	Cochrane
25	*T. harzianum*	Wood bark compost	Cochrane
26	*T. viride*	Wood bark compost	Cochrane
27	*T. viride*	Wood bark compost	Cochrane
28	*T. harzianum*	CDCS fungal culture collection	Brooks
29	*T. harzianum*	CDCS fungal culture collection	Brooks
30	*T. harzianum*	CDCS fungal culture collection	Brooks
31	*T. harzianum*	CDCS fungal culture collection	Brooks
32	*T. harzianum*	CDCS fungal culture collection	Brooks
33	*T. harzianum*	CCDS fungal culture collection	Brooks
34	*T. harzianum*	Mushroom compost	Crossfield
35	*T. harzianum*	Mushroom compost	Crossfield
36	*T. harzianum*	Mushroom compost	Crossfield
37	*T. harzianum*	Mushroom compost	Crossfield
38	*T. harzianum*	Mushroom compost	Crossfield

39	*T. harzianum*	Mushroom compost	Crossfield
40	*T. harzianum*	Mushroom compost	Crossfield
41	*T. harzianum*	Mushroom compost	Crossfield
42	*T. virens*	Mushroom compost	Crossfield
43	*T. harzianum*	Mushroom compost	Crossfield
44	*T. harzianum*	Mushroom compost	Edmonton
45	*T. rossicum*	Mushroom compost	Edmonton
46	*T. rossicum*	Mushroom compost	Edmonton
47	*T. rossicum*	Mushroom compost	Edmonton
48	*T. rossicum*	Mushroom compost	Edmonton
49	*T. rossicum*	Mushroom compost	Edmonton
50	*T. rossicum*	Mushroom compost	Edmonton
51	*T. rossicum*	Mushroom compost	Edmonton
52	*T. rossicum*	Mushroom compost	Edmonton
53	*T. rossicum*	Pumpkin plot soil	Brooks
54	*T. harzianum*	Pumpkin plot soil	Brooks
55	*T. rossicum*	Pumpkin plot soil	Brooks
56	*T. rossicum*	Pumpkin plot soil	Brooks
57	*T. rossicum*	Pumpkin plot soil	Brooks
58	*T. rossicum*	Pumpkin plot soil	Brooks
59	*T. rossicum*	Pumpkin plot soil	Brooks
60	*T. rossicum*	Pumpkin plot soil	Brooks
61	*T. rossicum*	Pumpkin plot soil	Brooks
62	*T. rossicum*	Pumpkin plot soil	Brooks
63	*T. rossicum*	Pumpkin plot soil	Brooks
64	*T. rossicum*	Alfalfa plot soil	Brooks
65	*T. rossicum*	Alfalfa plot soil	Brooks
66	*T. rossicum*	Rutabaga plot soil	Brooks
67	*T. harzianum*	Rutabaga plot soil	Brooks
68	*T. rossicum*	Rutabaga plot soil	Brooks
69	*T. harzianum*	Rutabaga plot soil	Brooks
70	*T. rossicum*	Rutabaga plot soil	Brooks
71	*T. rossicum*	Rutabaga plot soil	Brooks
72	*T. harzianum*	Herb plot soil	Brooks
73	*T. harzianum*	Herb plot soil	Brooks
74	*T. harzianum*	Herb plot soil	Brooks
75	*T. rossicum*	Herb plot soil	Brooks
76	*T. rossicum*	Herb plot soil	Brooks
77	*T. harzianum*	Herb plot soil	Brooks
78	*T. rossicum*	Herb plot soil	Brooks
79	*T. rossicum*	Herb plot soil	Brooks
80	*T. harzianum*	Herb plot soil	Brooks
81	*T. rossicum*	Herb plot soil	Brooks
82	*T. rossicum*	Herb plot soil	Brooks

83	*T. harzianum*	Herb plot soil	Brooks
84	*T. harzianum*	Herb plot soil	Brooks
85	*T. rossicum*	Herb plot soil	Brooks
86	*T. harzianum*	Herb plot soil	Brooks
87	*T. rossicum*	Herb plot soil	Brooks
88	*T. rossicum*	Herb plot soil	Brooks
89	*T. harzianum*	Bean root	Brooks
90	*T. harzianum*	Bean root	Brooks
91	*T. harzianum*	Bean root	Brooks
92	*T. harzianum*	Bean root	Brooks
93	*T. harzianum*	Tomato root	Lacombe
94	*T. harzianum*	Tomato root	Lacombe
95	*T. harzianum*	Tomato root	Lacombe
96	*T. harzianum*	Tomato root	Lacombe
97	*T. harzianum*	Spruce bark	Edmonton
98	*T. harzianum*	Cucumber leaf	Redcliff
99	*T. harzianum*	Cucumber leaf	Redcliff
100	*T. harzianum*	Sugar beet	Taber
101	*T. harzianum*	Sugar beet	Taber
102	*T. harzianum*	Sugar beet	Taber
103	*T. harzianum*	Turf grass root	Olds
104	*T. harzianum*	Bark compost	Cochrane
105	*T. harzianum*	Bark compost	Cochrane
106	*T. harzianum*	Bark compost	Cochrane
107	*T. harzianum*	Bark compost	Cochrane
108	*T. harzianum*	Bark compost	Cochrane
109	*T. harzianum* (T-22)	RootShield®	Commercial Product

66 strains of *T. harzianum*
33 strains of *T. rossicum*
8 strains of *T. virens*
2 strains of *T. viride*

Table 3-2. Co-culture assessment of *Trichoderma sp.* (strains 1 – 102) interaction with *Botrytis cinerea* (strain BN3) on PDA. Assessments were made at 1 and 2 weeks.

1. *Trichoderma* aggressive and fast gowing	*Trichoderma* sp. grew over *Botrytis cinerea* in 1 week and covered the entire Petri plate within 2 weeks. *Trichoderma* sp sporulation present where ever it grew over *B. cinerea*	5, 6, 9, 17, 18, 19, 21, 24, 26, 27, 29, 30, 33, 36, 42, 54, 93, 94, 95, 96, 97, 98, 99, 102
2. *Trichoderma* moderately aggressive and moderately fast growing.	After 1 week in culture, *Trichoderma* sp. was near but not growing over *B. cinerea*. *Trichoderma* sp. was growing within the interaction zone or over a portion of the *B. cinerea* colony within 2 weeks. *Trichoderma* sp. sporulation was evident where *Trichoderma* sp. grew over *B. cinerea*.	1, 8, 13, 23 25, 31, 32, 35, 38, 39, 40, 53, 55, 56, 59, 69, 72, 73, 74, 76, 77, 78, 80, 83, 84, 91, 92, 101
3. *Trichoderma* weak response.	*B. cinerea* and *Trichoderma* sp. show distinct regions of growth on the PDA medium. After 1 week a clear demarcation is evident at a zone of inhibition between the 2 species. No *Trichoderma* sp. growth over *B. cinerea* even after 2 weeks.	2, 3, 4, 7, 10, 11,12, 13, 14, 15, 16, 20, 22, 28, 34, 37, 41, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 57, 58, 60, 61, 62, 63, 64, 65, 66, 67, 68, 70, 71, 75, 79, 81, 82, 85, 86, 87, 88, 89, 90, 100

Table 3-3. Mean radial growth of *Botrytis cinerea* (LF5) after 1 week in Petri dish co-culture (PDA) with *Trichoderma* spp. strains 1 – 109 (n = 4).

***Trichoderma* spp. Strain**	**Radial growth (mm)**
B. cinerea control	72.0
81	34.0
59	33.8
56	32.8
57	32.8
58	32.5
55	32.2
62	32.0
61	32.0
78	31.2
79	31.2
60	30.8
80	30.8
64	30.8
96	30.5
88	30.0
18	29.8
70	29.8
43	29.5
85	29.5
5	29.2
9	29.2
42	29.2
83	29.0
22	29.0
17	29.0
86	29.0
41	29.0
108	28.8
39	28.8
40	28.8
103	28.8
106	28.8
101	28.8
68	28.5
46	28.5
38	28.5

35	28.2
75	28.2
95	28.2
84	28.2
19	28.2
92	28.2
15	28.2
8	28.2
87	28.2
82	28.0
53	28.0
36	28.0
89	28.0
50	28.0
52	28.0
6	28.0
24	28.0
102	28.0
63	27.8
7	27.8
23	27.8
44	27.8
20	27.8
104	27.8
77	27.5
2	27.5
105	27.2
25	27.2
31	27.2
66	27.2
65	27.0
73	27.0
107	27.0
94	27.0
47	27.0
1	26.8
74	26.8
12	26.8
16	26.8
72	26.8
45	26.5
13	26.5
71	26.5

100	26.5
33	26.5
109 (Rootshield strain T-22)	26.5
67	26.2
11	26.2
37	26.2
10	26.0
34	26.0
32	26.0
93	26.0
54	25.8
49	25.8
51	25.5
91	25.5
14	25.2
69	25.0
76	24.8
27	24.5
26	24.2
90	24.2
4	24.2
98	24.2
21	24.2
48	24.2
97	24.0
28	24.0
3	23.5
99	23.5
30	23.2
29	22.5

ANOVA Table

Excludes *B. cinerea* control from the analysis in order to concentrate on the differences in the strains of *Trichoderma* spp.

Source	D.F.	Type III S.S.	Mean Square	F value	Pr. > F
Strain	109	8407.25	70.65	7.92	<0.0001
Error	360	3210.75	8.92		

Table 3-4. Effect of petiole position on the mean lesion length on petioles colonized by *Botrytis cinerea* (LF5) on greenhouse tomato plants (cv. Quest, n = 8) 1 week after inoculation.

Petiole Position	**Lesion length (mm)**
Petiole position #1	47.6
Petiole position #2	46.7
Petiole position #3	45.0
Petiole position #4	43.7
Petiole position #5	42.2
Petiole position #6	41.7
Petiole position #7	41.5
Petiole position #8	37.5

Covariate Analysis, homogeneous variances– Compound Symmetry Model
Solution for Fixed Effects

Effect	**Estimate**	**Standard Error**	**D.F.**	**t value**	**Pr. > t**
Intercept	49.01	2.14	3	22.95	0.0002
Petiole	-1.28	0.42	27	-3.08	0.0048

Type 1 Test of Fixed Effects

Effect	**Numerator D.F.**	**Denominator D.F.**	**F value**	**Pr. > t**
Petiole	1	27	9.46	0.0048

Equation describing the linear relationship between lesion length and petiole position

$$y = 49.00 - 1.28 \text{ x petiole}$$

Table 3-5. Number of *Botrytis cinerea* conidia cm^{-2} on the terminal leaflets of the first fully expanded leaves of 35 tomato plants in a 120 m^2 greenhouse.

Plant Number	**Mean number of *B. cinerea* conidia cm^{-2}**
1	1.4×10^4
2	1.4×10^4
3	2.2×10^3
4	2.7×10^3
5	1.1×10^3
6	5.0×10^2
7	7.2×10^3
8	1.4×10^4
9	1.3×10^4
10	6.1×10^3
11	4.9×10^4
12	1.6×10^4
13	2.2×10^4
14	5.5×10^4
15	2.9×10^4
16	1.9×10^4
17	2.0×10^4
18	1.2×10^4
19	3.5×10^4
20	1.4×10^4
21	6.1×10^4
22	2.8×10^4
23	3.2×10^4
24	1.7×10^4
25	1.0×10^4
26	8.4×10^4
27	6.6×10^3
28	5.6×10^4
29	1.0×10^4
30	5.3×10^4
31	6.2×10^4
32	2.3×10^4
33	2.1×10^4
34	1.9×10^4
35	8.3×10^3
36	5.0×10^3

Table 3-6. Mean lesion lengths on petioles colonized by *Botrytis cinerea* (LF5) on greenhouse tomato plants (cv. Quest, n = 16) 1 week after co-inoculation with *Trichoderma* spp. strains 1–18.

***Trichoderma* spp. Strain**	**Lesion length (mm)**
B. cinerea control 33.1	
15	23.0
9	22.1
17	20.1
2	19.8
1	19.8
3	17.8
13	17.5
4	17.0
8	16.2
18	16.0
14	13.9
16	12.7
11	11.7
10	11.7
5	11.5
12	11.1
6	9.1
7	6.9
Negative control	0.0

ANOVA Table

Excludes both positive and negative controls from the analysis to concentrate on the differences among the strains of *Trichoderma* spp. All factors treated as random factors.

Source	**D.F.**	**Type III S.S.**	**Mean Square**	**F value**	**Pr. > F**
Strain	17	5777.18	339.83	1.40	0.2479
Date	1	404.18	404.18	1.66	0.2143
Date*Strain	17	4128.42	242.85	0.92	0.5476
Error	251	66055.00	263.17		

Table 3-7. Mean lesion lengths on petioles colonized by *Botrytis cinerea* (LF5) on greenhouse tomato plants (cv. Quest, n = 16) 1 week after co-inoculation with *Trichoderma* spp. strains 19-48.

***Trichoderma* spp. Strain**	**Lesion length (mm)**
37	38.4
B. cinerea control	36.3
28	35.8
48	35.7
36	35.1
33	34.9
35	34.8
31	32.2
32	31.4
47	29.8
34	28.0
43	18.8
45	18.4
26	17.7
40	17.2
46	16.8
41	15.9
38	14.6
25	14.3
20	13.5
30	13.3
24	13.0
22	13.0
39	12.4
19	12.3
27	11.4
23	11.2
21	10.8
42	10.5
44	10.4
29	4.9
Negative control	0.0

ANOVA Table

Excludes both positive and negative controls from the analysis to concentrate on the differences among the strains of *Trichoderma* spp. All factors treated as random factors.

Source	**D.F.**	**Type III S.S.**	**Mean Square**	**F value**	**Pr. > F**
Strain	29	48044.00	1656.70	4.18	0.0001
Date	1	10027.00	10027.00	25.32	<0.0001
Date*Strain	29	11490.00	396.19	2.58	<0.0001
Error	417	64116.00	153.76		

Table 3-8. Mean lesion lengths on petioles colonized by *Boytris cinerea* (LF5) on greenhouse tomato plants (cv. Quest, n = 16) 1 week after co-inoculation with *Trichoderma* spp. strains 49–68.

***Trichoderma* spp. Strain**	**Lesion length (mm)**
B. cinerea control	31.2
51	25.4
55	25.3
59	24.1
62	23.4
64	22.9
53	22.1
49	21.9
60	21.4
67	21.0
57	20.7
61	20.4
65	20.0
68	19.6
54	19.4
52	19.1
50	18.4
66	18.3
56	17.9
63	17.3
58	15.3
Negative control	0.0

ANOVA Table

Excludes both positive and negative controls from the analysis to concentrate on the differences among the strains of *Trichoderma* spp. All factors treated as random factors.

Source	D.F.	Type III S.S.	Mean Square	F value	Pr. > F
Strain	19	2107.95	110.94	0.71	0.7729
Date	1	28294.00	28294.00	179.90	<0.0001
Date*Strain	19	2887.92	157.26	0.80	0.7020
Error	278	54377.00	195.60		

Table 3-9. Mean lesion lengths on petioles colonized by *Botrytis cinerea* (LF5) on greenhouse tomato plants (cv. Quest, n = 16) 1 week after co-inoculation with *Trichoderma* spp. strains 69–88.

***Trichoderma* spp. Strain**	**Lesion Length (mm)**
B. cinerea control	36.2
78	30.8
74	30.2
70	26.9
80	26.9
76	26.1
79	24.7
81	24.6
84	24.0
88	23.9
71	23.6
82	23.4
69	23.4
75	22.6
85	21.7
72	21.1
86	21.0
87	20.6
73	20.1
77	16.9
83	16.8
Negative control	0.0

ANOVA Table

Excludes both positive and negative controls from the analysis to concentrate on the differences among the strains of *Trichoderma* spp. All factors treated as random factors.

Source	**D.F.**	**Type III S.S.**	**Mean Square**	**F value**	**Pr. > F**
Strain	19	4196.62	220.87	1.50	0.1915
Date	1	27787.00	27787.00	188.96	<0.0001
Date*Strain	19	2793.67	147.04	0.65	0.8611
Error	279	62630.00	224.48		

Table 3-10. Mean lesion lengths on petioles colonized by *Boytris cinerea* (LF5) on greenhouse tomato plants (cv. Quest, n = 16) 1 week after co-inoculation with *Trichoderma* spp. strains 89–109.

***Trichoderma* spp. Strain**	**Lesion Length (mm)**
B. cinerea control	35.9
106	21.8
102	20.9
94	19.2
103	18.3
104	17.9
95	17.1
100	17.1
91	16.9
98	16.9
96	16.6
109 (Rootsheild strain T-22)	15.9
107	15.8
92	15.1
97	13.8
90	13.8
101	12.4
89	11.1
99	10.6
93	9.3
105	6.4
108	6.0
Negative control	0.0

ANOVA Table

Excludes both positive and negative controls from the analysis in to concentrate on the differences among the *Trichoderma* spp. strains. All factors treated as random factors.

Source	**D.F.**	**Type III S.S.**	**Mean Square**	**F value**	**Pr. > F**
Strain	20	5898.23	294.91	1.30	0.2818
Date	1	92.61	92.61	0.41	0.5302
Date*Strain	20	4539.54	226.98	1.00	0.4603
Error	292	66183.00	226.66		

Table 3-11. Mean lesion lengths on tomato petioles colonized by *Boytris cinerea* (LF5) on greenhouse tomato plants (cv. Quest, n = 4) 1 week after co-inoculation with *Trichoderma* spp. strains 7 and 29 in the third greenhouse evaluation of these strains.

Treatment	**Lesion length (mm)**	
B. cinerea control	44.7	a
108	37.2	ab
99	24.0	ab
105	22.7	ab
18	22.2	ab
93	22.2	ab
7	19.5	ab
29	10.5	b

Numbers followed by the same letter are not significantly different ($P \leq 0.05$) as determined by the Tukey Test.

Table 3-12. Effect of spray and droplet application (10 µL droplet) of *Trichoderma harzianum* strains 7 and 29 at 10^{7} spores mL^{-1} spore suspension on the mean lesion length on tomato petioles caused by *Botrytis cinerea* (LF5) on greenhouse tomato plants (cv. Quest, n = 8) one week after co–inoculation. *B. cinerea* applied via a 10 µL droplet application at 10^{6} spores mL^{-1}.

Treatment	**Lesion length (mm)**
B. cinerea followed by water spray	42.2
B. cinerea followed by water droplet	28.2
B. cinerea followed by *T. harzianum* 29 droplet application	10.2
B. cinerea followed by *T. harzianum* 7 droplet application	5.1
B. cinerea followed by *T. harzianum* 29 spray application	3.1
B. cinerea followed by *T. harzianum* 7 spray application	0.2

ANOVA Table
Type III Tests of Fixed Effects

Effect	**Numerator D.F.**	**Denominator D.F.**	**F value**	**Pr. > F**
Strain (includes *B. cinerea* control)	2	42	26.69	< 0.0001
Application method	1	42	0.06	0.8070
Strain *Application	2	42	2.49	0.0949

Estimates

	Estimate	**Standard Error**	**D.F.**	**t value**	**Pr. > t**
T. harzianum strains vs. positive control	-31.4712	4.60	42	-6.84	<0.0001
Strain 29 vs. strain 7	-4.00	2.71	42	-1.48	0.1476

95% Confidence interval on the difference between *T. harzianum* strains 29 and 7 and the positive control.

= Y≤ t (0.05,42) x 4.602 = 31.47≤ 2.018 x 4.602 = **31.47 ≤9.32 mm**

Table 3-13. Effect of spray application of *Trichoderma harzianum* spore suspension (strains 7 and 29 at 10^{7} spores mL^{-1}) at different times before and after inoculation with *B. cinerea* (LF5 at 10^{4} spores mL^{-1}) on the mean lesion length on tomato petioles (cv. Quest, n = 4) colonized by *Botrytis cinerea* 1 week after inoculation.

Treatment	**Lesion Length (mm)**
B. cinerea inoculated followed by water after 2 hours	47.2
B. cinerea inoculated followed by water after 3 hours	46.5
Water application followed by *B. cinerea* after 1 hour	46.0
Water application followed *by B. cinerea* after 2 hours	42.2
B. cinerea inoculated followed by water after 1 hour	40.8
Simultaneous application of *B. cinerea* and water	36.5
Water application followed by *B. cinerea* after 3 hours	26.5
T. harzianum 7 inoculated followed by *B. cinerea* after 1 hour	7.2
Simultaneous application of *B. cinerea* and *T. harzianum* 7	6.8
T. harzianum 7 inoculated followed by *B. cinerea* after 3 hours	4.7
Simultaneous application of *B. cinerea* and *T. harzianum* 29	2.5
T. harzianum 29 inoculated followed by *B. cinerea* after 3 hours	1.5
B. cinerea inoculated followed by *T. harzianum* 7 after 1 hour	1.5
T. harzianum 7 inoculated followed by *B. cinerea* after 2 hours	1.0
T. harzianum 29 inoculated followed by *B. cinerea* after 2 hours	0.5
B. cinerea inoculated followed by *T. harzianum* 29 after 1 hour	0.2
T. harzianum 29 inoculated followed by *B. cinerea* after 1 hour	0.2
B. cinerea inoculated followed by *T. harzianum* 7 after 3 hours	0.2
B. cinerea inoculated followed by *T. harzianum* 7 after 2 hours	0.0
B. cinerea inoculated followed by *T. harzianum* 29 after 2 hours	0.0
B. cinerea inoculated followed by *T. harzianum* 29 after 3 hours	0.0

ANOVA Table

Type III Tests of Fixed Effects

Effect	**Numerator D.F.**	**Denominator D.F.**	**F value**	**Pr. > F**
Strain (includes *B. cinerea* control)	2	63	146.10	< 0.0001
Time of application	6	63	0.54	0.7726
Strain *Time	12	63	1.07	0.6568

Table 3-14. Comparison of the effect of fungicides to application of *Trichoderma harzianum* strains 7 and 29 on the mean lesion length on petioles colonized by *Botrytis cinerea* (LF5) on greenhouse tomato plants (cv. Quest, n = 8) 1 week after application.

Treatment	**Lesion length (mm)**	
B. cinerea control	27.0	a
Benomyl + Mancozeb	9.5	ab
T. harzianum 7	5.1	b
T. harzianum 29	5.5	b
Iprodione	1.6	b

Numbers followed by the same letter are not significantly different ($P \leq 0.05$) as determined by the Tukey Test.

Orthogonal Contrasts	**P value**
Control vs. others	0.0002
Biocontrol vs fungicides	0.8384
Strain 7 vs. strain 29	0.7585
Iprodione vs. Benomyl + Mancozeb	0.2305

Table 3-15. Second comparison of the effect of fungicides to application of *Trichoderma harzianum* strains 7 and 29 on the mean lesion length on tomato petioles colonized by *Botrytis cinerea* (LF5) on greenhouse tomato plants (cv. Quest, n= 8) 1 week after application.

Treatment	**Lesion Length (mm)**	
B. cinerea control	28.5	a
Benomyl + Mancozeb	12.5	ab
Captan	8.9	ab
Azoxystrobin	5.9	b
T. harzianum #7	5.1	b
Iprodione	4.4	b
Fenhexamid	4.2	b
T. harzianum #29	3.2	b

Numbers followed by the same letter are not significantly different ($P \leq 0.05$) as determined by the Tukey Test.

Orthogonal Contrasts	**P value**
Control vs. others	< 0.0001
Biocontrol vs fungicides	0.4499
Strain 7 vs. strain 29	0.7775
Newer vs. Older fungicides (Fenhexamide, Azoxystrobin, Iprodione vs. Benomyl +Manzate, Captan)	0.4208

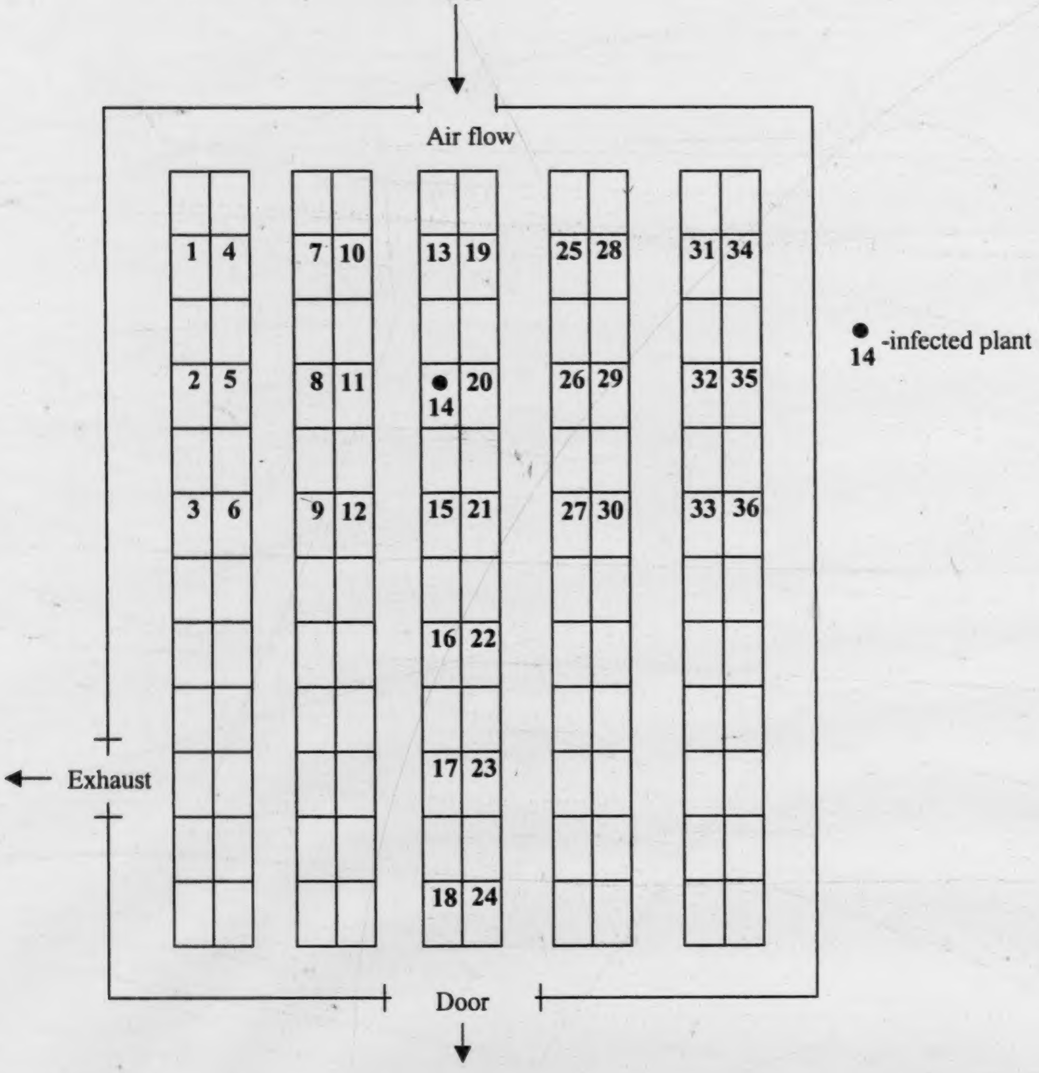
Air flow
1
4
7
10
13
19
25
28
31
34
2
5
8
11
14
20
26
29
32
35
3
6
9
12
15
21
27
30
33
36
16
22
17
23
18
24
Exhaust
Door
● 14 -infected plant

Figure 3-1. Schematic diagram of a research greenhouse with tomato plants indicating the positions of the 36 plants examined for *Botrytis cinerea* conidia after 1 sporulating stem lesion was found (plant 14).

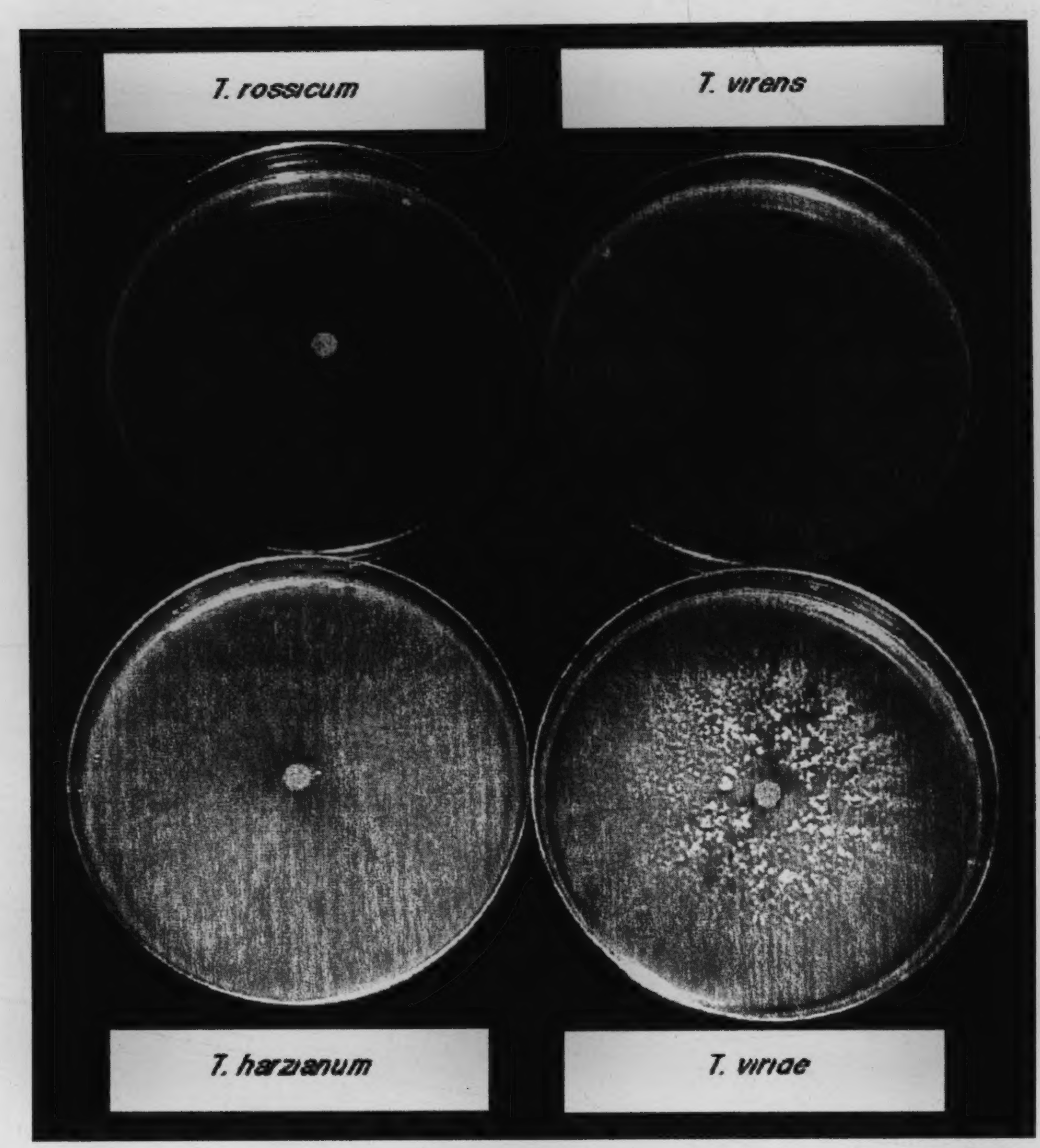
T. rossicum
T. virens
T. harzianum
T. viride

Figure 3-2. Colony morphology of 4 day-old cultures of representative strains of *Trichoderma rossicum* (strain 82), *Trichoderma virens* (strain 18), *Trichoderma harzianum* (strain 7) and *Trichoderma viride* (strain 27) on malt extract agar

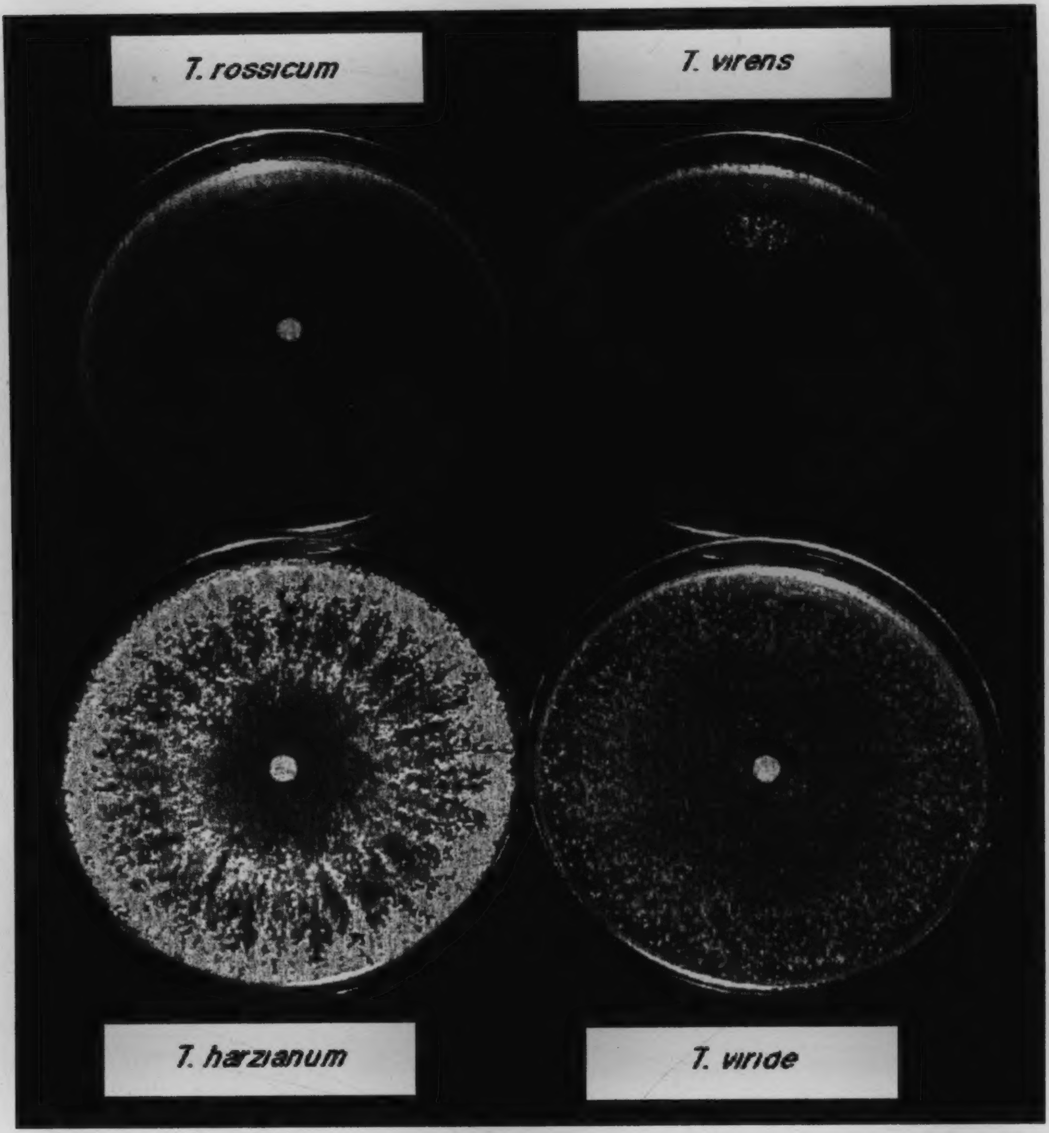
T. rossicum
T. virens
T. harzianum
T. viride

Figure 3-3. Colony morphology of 6 day-old cultures of representative strains of *Trichoderma rossicum* (strain 82), *Trichoderma virens* (strain 18), *Trichoderma harzianum* (strain 7) and *Trichoderma viride* (strain 27) on malt extract agar

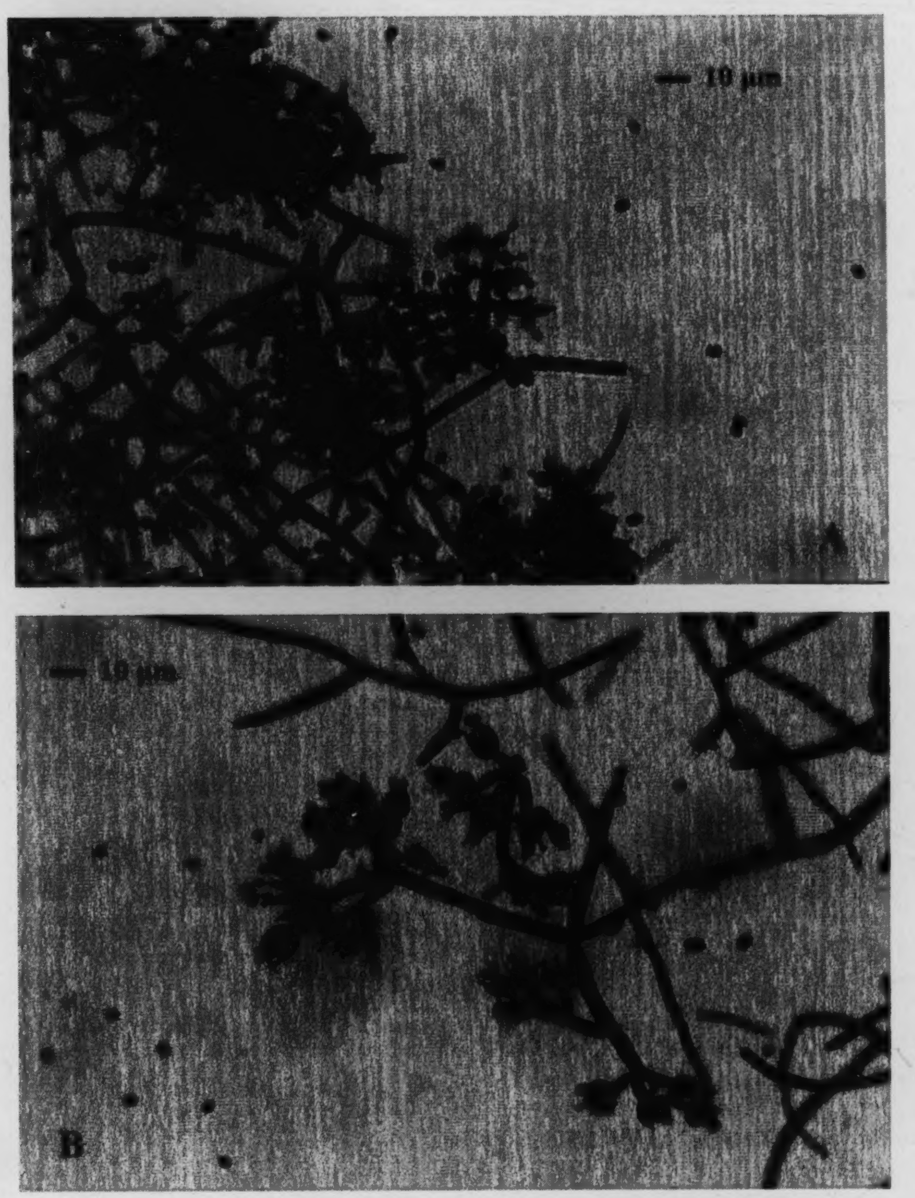

Figure 3-4. Phialide and conidal morphology of representative strains of (A) *Trichoderma rossicum* (strain 82) and (B) *Trichoderma virens* (strain 18), from 4 day-old cultures grown on malt extract agar

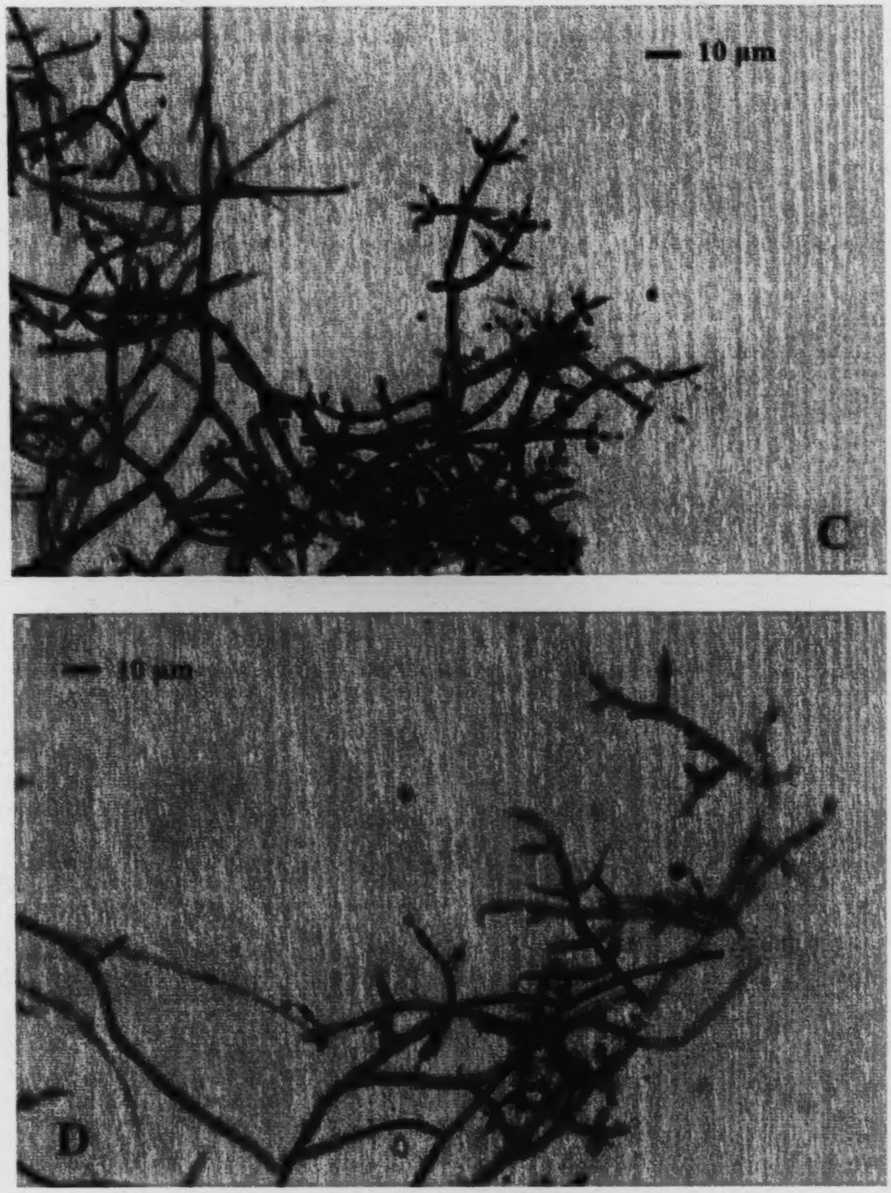

Figure 3-5 Phialide and conidal morphology of representative strains of (C) *Trichoderma harzianum* (strain 7) and (D) *Trichoderma viride* (strain 27), from 4 day-old cultures grown on malt extract agar

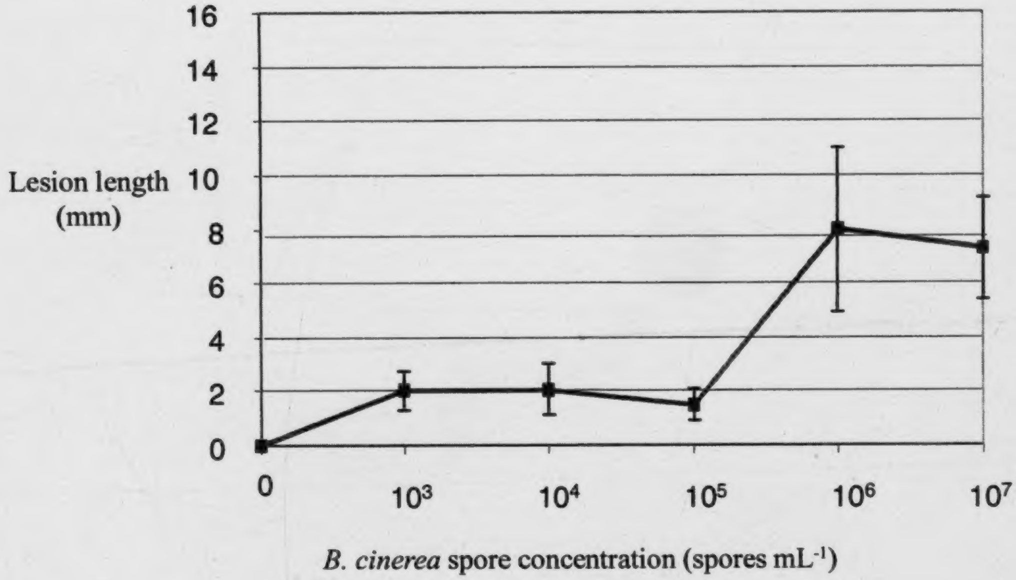

Figure 3-6. Mean lesion lengths, including standard error, on tomato petioles (cv. Quest, n = 4) colonized by *Boytris cinerea* isolate LF5 with increasing levels of spore concentration. Measurements were recorded 1 week after inoculation.

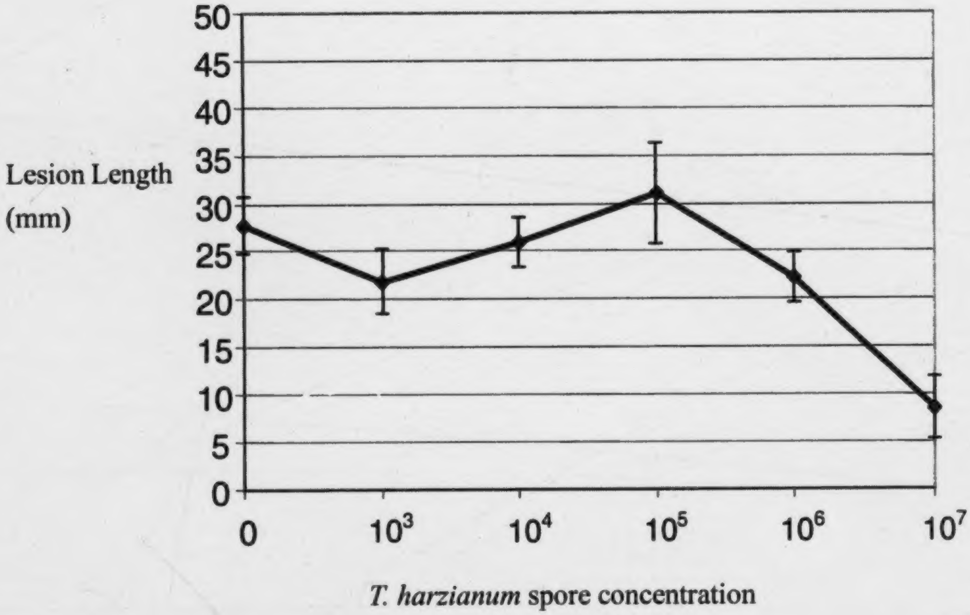

Figure 3-7. Mean lesion lengths, including standard error, on tomato petiole (cv. Quest, n = 4) colonized by *Botrytis cinerea* isolate LF5 after co-inoculation with increasing levels of spore concentration of *Trichoderma. harzianum* strain 7. Measurements were recorded 1 week after co-inoculation.

Figure 3-8. Tomato petiole bioassay, negative control (no *Botrytis cinerea*, no *Trichoderma* sp.) after 1 week.

Figure 3-9. Tomato petiole bioassay positive control (*Botrytis cinerea* only) after 1 week.

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Chapter 4

RAPD PCR analysis of strains of *Trichoderma harzianum* and *Botrytis cinerea*[1].

4.1 Introduction

Random amplified polymorphic DNA (RAPD) PCR (polymerase chain reaction) analysis (Welsh and McClelland 1990) provides a method to assess genetic variation and relatedness between and within species. Comparisons between polymorphisms in genomic fingerprints produced by the RAPD method is sufficient to distinguish between strains and species (Welsh and McClelland 1990). No prior knowledge of genetic information of the species is required (Welsh and McClelland 1990; Weising *et al.* 1995).

The RAPD technique has proven to be a reliable procedure. For example in genetic comparisons among strains of *T. harzianum*, this technique gave results similar to those obtained using restriction fragment length polymorphisms (RFLPs) (Muthumeenakshi *et al.* 1998).

The RAPD technique was used to distinguish between strains and species of *Trichoderma* and distinguished the biologicial control strain of *T. harzianum* (T-39) from other strains of *T. harzianum* (Zimand *et al.*1994). RAPD analysis was also used to distinguish aggressive strains of *T. harzianum* from mushroom compost (Muthumeenakshi *et al.* 1998). This technique is also able to distinguish strains of *T. viride* having biological control activity against the chestnut blight pathogen *Cryphonectria parasitica* (Murrill) Barr (Arisan-Atac *et al.*1995). Use of the RAPD technique was suggested as a convenient method for screening a large number of strains for biological control potential of *Trichoderma* spp. (Arisan-Atac *et al.*1995).

RAPD analysis has also been applied to investigate the variability among strains of *B. cinerea* collected inside and outside of a greenhouse in the Netherlands. No correlation was found among pathogenicity, sampling date or sampling location, and the RAPD patterns. The authors concluded that *B. cinerea* inoculum in greenhouses continuously originate from many sources in the vicinity (Kerssies and Bosker-van Zessen 1997). Another RAPD study of greenhouse populations of *B. cinerea* reported a lack of differentiation of *B. cinerea* collected from 23 greenhouses sampled from 2 different regions in south-eastern Spain (Alfonso *et al.* 2000).

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[1] A version of this Chapter has been published: Calpas, J.T., Konschuh, M.N., Towes, C.C., and Tewari, J.P. 1999, Can. J. Plant Pathol. 23: 184. [abstract].

RAPD characterization of *B. cinerea* collected from table grapes in Chile suggested host-pathogen specialization (Thompson and Latorre 1999). Strains collected from table grapes grouped together, strains collected from apple and tomato formed a second group, and strains from blueberry formed the third group (Thompson and Latorre 1999).

Another study from France also determined that a degree of host specialization may exist within strains of *B. cinerea* associated with the presence or absence of transposable elements within the strains (Giraud *et al.* 1997; Giraud *et al.* 1999). *Botrytis cinerea* strains containing the transposable elements *Boty* and *Flipper* were named *transposa* strains and those strains without the transposable elements named *vacuma* strains (Giraud *et al.* 1997). *Transposa* strains were suggested to be more adapted to infecting grapes than the *vacuma* strains (Giraud *et al.* 1997). No differentiation between strains was detected from different locations with *transposa* and *vacuma* and the strains were considered as sympatric (Giraud *et al.* 1997).

Subsequent work in France confirmed that even though *transposa* and *vacuma* strains were sometimes found on the same host plants, their relative prevalence were significantly different (Giraud *et al.* 1999). *Transposa* and *vacuma* strains did not display the same ability to infect different hosts with no apparent geographic differentiation (Giraud *et al.* 1997; Giraud *et al.* 1999). The presence of *transposa* and *vacuma* strains were confirmed in *B. cinerea* populations in Chile and RAPD analysis revealed a significant host effect (Muñoz *et al.* 2002). Strains of *B. cinerea* collected from the same host plants tended to cluster together regardless of their geographical origin (Muñoz *et al.* 2002).

Fournier *et al.* (2003) characterized *B. cinerea* strains into 2 groups (Group I and II) based on the presence of 2 alleles of the Bc-*hch* gene associated with vegetative incompatibility in *B. cinerea*. These groups did not correspond to the groups observed by Giraud *et al.* (1997). Fournier *et al.* (2003) observed that Group I and II strains were not able to form sexual crosses, both groups were considered genetically isolated. They also reported that Group I and II strains were vegetatively incompatible and that a number of vegetative compatibility groups were present within the Group II strains.

Knowledge of the population genetics of *B. cinerea* may contribute to the development of more effective disease management strategies (Alfonso *et al.* 2000). The existence of host specialization in *B. cinerea* could lead to management strategies that include crop rotation. Similarly, knowledge of the genetics of *T. harzianum* may contribute to the development of techniques to identify and select more effective biological control strains.

This is the first Canadian study to conduct an extensive investigation into the population genetics of Alberta strains of *B. cinerea* in greenhouse crops and is the first study to characterize biological control potential of Alberta strains of *T. harzianum* against *B. cinerea*

The objectives of this study were;

1. To assess the genetic diversity of strains of *T. harzianum* with respect to the biological control potential against *B. cinerea*.

2. To assess the genetic diversity of strains of *B. cinerea* with respect to host specificity and collection location.

4.2 Materials and methods

4.2.1 Selection of strains

4.2.1.1 *Trichoderma harzianum*

A subset of 12 *T. harzianum* strains were selected based on their relative biological control activity as determined by the greenhouse tomato petiole bioassay over all 5 trials (Chapter 3). The commercial RootShield® strain of *T. harzianum*, T-22 was included for comparison. The selected strains demonstrated consistent "better" or "poorer" biological control activity in both repeats of the tomato petiole bioassay.

Trichoderma harzianum strains 15, 2, 74 and 80 were selected to represent the relatively poor performing biological control strains while strains 7, 29, 39, 23, 44, 99 and 105 represented the better performing strains. The RootShield® strain T-22 was a reasonably good preforming strain.

4.2.1.2 *Boytris cinerea*

Four subsets of *B. cinerea* were selected from the 160 strains described in Chapter 2 and examined separately. The first subset was comprised of all 38 *B. cinerea* strains collected from greenhouse tomato plants. This subset was selected in order to examine the genetic diversity among strains collected from the same host plant species from a variety of greenhouse locations. The second subset was made up of 50 strains collected from a variety of host plant species from 10 greenhouse locations. This subset was selected in order to examine genetic diversity among strains collected from a number of host plant species from different greenhouse locations.

The third subset included 35 strains from 31 locations, 23 greenhouse and 8 field locations. One strain was included from each location with the exception of 3 strains selected from both greenhouse locations LF (3 tomato strains) and EO (3 cucumber strains). This subset was selected to examine the genetic diversity among strains collected from a number of hosts from a variety of locations primarily represented by one strain. The fourth subset included 35 strains collected from a number of host plants that were also assessed for virulence against cucumber, petunia, and geranium in stem piece bioassays (Chapter 2). This subset was selected to examine the genetic diversity among strains displaying differential virulence across four host plant species.

4.2.2 DNA extraction

The DNA extraction method was similar to that of Weising et al. (1995), and was used to extract DNA from the strains of *B. cinerea* and *T. harzianum*.

A single 5 mm agar (PDA) plug of each *B. cinerea* and *T. harzianum* strain was transferred to individual, sterile, 39 mL flat-bottomed Pyrex® culture tubes containing 10 mL of potato dextrose broth (PDB) (Difco). The liquid cultures were kept at 20°C on a shaker (120 rpm) for 3 days under ambient light. Between 0.1 to 0.3 g of mycelium (fresh weight) was taken from the liquid cultures at day 3 and transferred to sterile 1.5 mL microfuge tubes and centrifuged (14,000 x g) for 5 min. The culture medium was removed, and the mycelium was rinsed with 1X TE buffer (10 mM Tris-HCL, pH 8.0, and 1 mM EDTA) and then centrifuged as before. The TE buffer was removed and the mycelium was homogenized in 500 µL of DNA extraction buffer (200 mM Tris-HCL, pH 8.5, 250 mM NaCl, 25 mM EDTA, and 0.5% SDS) with a disposable microfuge pestle. Liquid nitrogen was added to freeze the mycelium and a small amount of aluminum oxide was also added to aid in grinding and cell disruption. The homogenized samples were centrifuged (5,000 x g) for 5 min and the supernatant was treated with 50 µL RNAase A for 1 h at 37°C. Samples were then extracted twice with an equal volume of phenol:choroform:isoamyl alcohol (25:24:1) and then once with chloroform:isoamyl alcohol (24:1). Sodium acetate (3M) (250 µL) was added to the aqueous phase in a 1.5 mL tube. The samples were incubated for 10 min at –20°C and then centrifuged (14,000 x g) for 5 min. DNA was precipitated at room temperature from the clarified supernatant by the addition of 750 µL isopropanol and an incubation of at least 10 min. The samples were centrifuged (14,000 x g) for 15 min to collect the DNA pellet, the pellet was washed with 70% ethanol, and then resuspended in 50 – 250 µL 1X TE buffer by heating at 55°C. An aliquot of each DNA sample was run on a 1% agarose gel to check the integrity and yield. Isolated DNA was stored at –20°C until used for RAPD analysis.

4.2.3 RAPD-PCR analysis

4.2.3.1 RAPD-PCR analysis of *Botrytis cinerea*

The extracted *B. cinerea* DNA was used to conduct the PCR protocol for RAPD analysis of *B. cinerea*. The protocol was optimized with respect to enzyme concentration, primer concentration, $MgCl_2$ concentration, number of amplification cycles, and primer annealing temperatures. Polymerase chain reactions were carried out in thin walled 0.5 mL microcentrifuge tubes in a total volume of 25.00 µL. Each reaction contained 17.65 µL sterile water, 2.50 µL 10X PCR buffer (200 mM Tris-HCl, pH 8.4, 500 mM KCl; Gibco BRL, Gaithersburg, MD), 0.50 µL 10 mM dNTP's, 1.25 µL, 50 mM $MgCl_2$, 0.10 µL Taq Polymerase (5 U/ L; Gibco BRL), 1.00 µL (10 pmol) of primer, and 2.00 µL (50 ng) genomic DNA. The reactions were amplified in a PTC-100 Thermocycler (M.J. Research, Inc., Watertown, MA). After an initial denaturation at 94°C, DNA was amplified for 45 cycles of 15 s at 94°C, 45 s at 36°C, and 90 s at 72°C. The final extension was continued for 4 min at 72°C and then held at 4°C until the products were separated by gel electrophoresis. A 5 µL aliquot of amplified DNA was separated by electrophoresis on a 1.25% agarose gel in 0.5X TBE (Tris-borate-EDTA) buffer. Gels were stained with ethidium bromide to visualize the DNA under UV light. Photographs were taken using a Photoview gel documentation system (Bio/Can Scientific, Mississauga, ON) and Type 667 black and white Polaroid film (Polaroid Corp., Cambridge, MA). The size of separated bands was determined by comparison with a 1 kb DNA ladder (Gibco BRL).

Botrytis cinerea DNA was amplified by the RAPD method using the "A", "B", and "D" kits of random oligonucleotide (10-mer) primers from Operon Technologies, Inc. (Alameda, CA). A total of 60 RAPD primers were used to produce polymorphic bands between 7 strains of *B. cinerea* (strains LF1, EC1 PR1, K1, WD1, ST1, SB1) The 10 primers used for this study were selected based on the number of clear and reproducible bands amplified from the DNA samples and the production of polymorphic bands that distinguished between the 7 different *B. cinerea* strains. To ensure that amplification products were not primer artifacts, genomic DNA was omitted from a control reaction for each primer examined. PCR amplifications were repeated once.

4.2.3.2 RAPD-PCR analysis of *Trichoderma harzianum*

The extracted *T. harzianum* DNA was used to conduct the PCR protocol for RAPD analysis of *T. harzianum.* Polymerase chain reactions were carried out in thin walled 0.5 mL microcentrifuge tubes in a total volume of 25.00 µL. The PCR protocol used for *B. cinerea* was followed for *T. harzianum* with the following exceptions. Each reaction contained 16.65 µL sterile water, 2.50 µL 10X PCR buffer (200 mM Tris-HCl, pH 8.4, 500 mM KCl; Gibco BRL, Gaithersburg, MD), 0.50 µL 10 mM dNTP's, 1.25

µL 50 mM $MgCl_2$, 0.10 µL Taq Polymerase (5 U/ L; Gibco BRL), 2.00 µL (10 pmol) of primer, and 2.00 µL (50 ng) genomic DNA.

T. harzianum DNA was amplified by the RAPD method using the "A", "B", "D" and "E" kits of random oligonucleotide (10-mer) primers from Operon Technologies, Inc. (Alameda, CA). A total of 80 RAPD primers were used to produce polymorphic bands between 6 *Trichoderma* spp. strains (strains 6, 27, 34, 38, 45, 76). The 10 primers used for this study were selected based on the number of clear and reproducible bands amplified from the DNA samples and the production of polymorphic bands that distinguished between the 6 different *T. harzianum* strains. To ensure that amplification products were not primer artifacts, genomic DNA was omitted from a control reaction for each primer examined. PCR amplifications were repeated once.

4.3 Statistical analyses of RAPD data

The RAPD fingerprints generated by the different primers were compared for their relatedness within the separate groups of *B. cinerea* and *T. harzianum* strains. Reproducible amplification fragments (amplicons) between 300 and 2,500 base pairs for each primer were identified and scored for presence or absence in the populations. Occasionally, some primers did not generate amplification fragments in the higher molecular weight range with some samples. In those cases, all samples were scored relative to the most limited sample.

Dissimilarity values were computed from all pairwise population comparisons of the RAPD data and used to calculate genetic distance. Dissimilarity values (genetic distances) were calculated using the formula $GD = 1 - [2N_{ab}/(n_a + n_b)]$, where GD = genetic distance; N_{ab} = the number of shared amplicons with the same molecular weight between populations a and b; n_a = the number of scored amplicons for population a, and n_b = the number of scored amplicons for population b (Nei and Li 1979). Dissimilarity was used as a measure for genetic distance as it compensates for instances where comigrating amplicons represent different sequences (Shinners and Tewari 1998). Dissimilarity values ranged from 0 to 1, with 1 being dissimilar and 0 being similar.

The dissimilarity values were used to construct a matrix, with the data subjected to UPGMA (unweighted paired group method of arithmetic averages) analysis using the SAHN program in the NTSYSpc 2.0 software package (Exeter Software, Setauket, New York) to produce a phenogram. All tied phenograms were accounted for (Beackeljau et al. 1996), and the goodness of fit of the dendrogram to the matrix data was calculated as a cophenetic correlation coefficient using the MXCOMP program in NTSYSpc.

4.4 Results

4.4.1 RAPD analysis of *Trichoderma harzianum*

The 10 RAPD primers produced a total of 219 clear, reproducible polymorphic bands between 500 and 3,000 bp from the 12 *T. harzianum* strains (Table 4-1) and the resulting phenogram is presented in Figure 4-1. The clustering analysis resulted in 3 clearly defined groups. The first branch contains one cluster consisting of strains 44, T-22, 29 and 99 (Group 1) separated from the other strains by a genetic distance of 0.80. The other branch contains the majority of the strains, and further branched into 2 clusters (Groups 2 and 3) separated by a genetic distance of 0.63. The strains in Group 1 all exhibited better biological control performance against *B. cinerea* as assessed using the tomato petiole bioassay. The Group 2 strains also were characterized as better biological control strains. Group 3 contained all the poorer biological control strains with the exception that the better biological control strains 7 and 105 were also included in this group.

All of the "poorer" biological control strains grouped together, with the majority of the "better" biological control strains grouping separately demonstraing more than 75% dissimilarity to the poorer strains.

4.4.2 RAPD analysis of *Botrytis cinerea*

4.4.2.1 RAPD analysis of 38 strains of *Botrytis cinerea* collected from greenhouse tomato in Alberta

The 10 RAPD primers produced a total of 124 clear, reproducible polymorphic bands between 500 and 3,000 bp from the 38 strains of *B. cinerea* (Table 4-2). Banding patterns were more similar between strains from the same locations and more distinct between strains collected from different locations. The comparison of RAPD patterns of 15 tomato strains collected from 3 locations (LF, BT and TM) generated by primer OPB01 is presented in Figure 4-6. A polymorphic band at 1400 bp, is present in the TM strains but is absent in all the LF and BT strains. The BT strains all have bands at 800 and 1000 bp with variability in the presence of the bands at 1700 and 1800 bp. The polymorphic band at 800 bp is present in all BT and TM strains and strains LF2, LF4 and LF5, but is absent in strains LF1 and LF3. Strains LF3 and LF4 also have a band at 1300 bp that is not present in any of the other strains.

The resulting phenogram is presented in Figure 4-2. The clustering analysis resulted in 8 clearly defined groups corresponding to the greenhouse locations from which the strains were collected. All of the strains were within a maximum genetic distance of 0.397, with a range between groups of 0.05 (Brooks, TM strains) to 0.22 (Strathmore, BT strains).

4.4.2.2 RAPD analysis of 50 strains of *Botrytis cinerea* collected from a number of host plant species from 10 greenhouse locations in Alberta

The 10 RAPD primers produced a total of 130 clear, reproducible polymorphic bands between 500 and 3,000 bp from the 50 strains of *B. cinerea* (Table 4-3) and the resulting phenogram is presented in Figure 4-3. The clustering analysis resulted in 10 clearly defined groups corresponding to the greenhouse locations from which the strains were collected. No clustering according to host species is evident. Strains collected from different host species clustered together based on collection location. All of the strains were within a maximum genetic distance of 0.380, with a range between groups of 0.061 (Edmonton, WL strains) to 0.167 (Bragg Creek, EO strains).

4.4.2.3 RAPD analysis of 35 strains of *Botrytis cinerea* collected from a variety of host plant species from 31 greenhouse and field locations in Alberta

The 10 RAPD primers produced a total of 135 clear, reproducible polymorphic bands between 500 and 3,000 bp from the 35 strains of *B. cinerea* (Table 4-4) and the resulting phenogram is presented in Figure 4-4. Cluster analysis resulted in 2 clearly defined groups. The first group included strains LF1, LF4, and LF5 all collected from tomato in one greenhouse located in Strathmore. The second group included the strains EO1, EO2, and EO3, all collected from cucumber in one greenhouse located in Bragg Creek. All of the 35 strains were within a genetic distance of 0.399. The 3 LF strains were within a genetic distance of 0.094, the 3 EO strains were within a genetic distance of 0.147.

Other clusters of 3 strains are also present, however, there is no correlation with host species. The only clusters that display a correlation with host species are the 3 LF strains and the 3 EO strains, collected from tomato and cucumber, respectively. The results presented in Figures 4-2 and 4-3 demonstrates that these strains cluster together because of collection location rather than host species. Similarly, the lack of cluster formation based on host species for any other strains in Figure 4-4, supports that the LF and EO strains cluster together due to the effect of collection location.

4.4.2.4 RAPD analysis of 35 strains of *Boytris cinerea* collected from a number of host plant species and assessed for virulence against tomato, cucumber, geranium, and petunia in stem piece bioassays

The 10 RAPD primers produced a total of 145 clear, reproducible polymorphic bands between 500 and 3,000 bp from the 35 strains of *B. cinerea* (Table 4-5) and the resulting phenogram is presented in Figure 4-5. The bold letters preceding the strain identification on the phenogram indicate strains that were characterized as virulent on tomato, cucumber, petunia or geranium. Strains were characterized as virulent on a particular host when the strain colonized greater than 15 mm of host stem in one week as determined by stem piece bioassays.

Cluster analysis did not result in groups that were based on virulence on specific hosts or virulence on a number of host species. The 35 strains were within a genetic distance of 0.382. The 3 LF and 3 EO strains were also included in this group of 35 strains and these strains formed the only clearly identifiable groups as in the previous assessment. The LF strains were within a genetic distance of 0.089, and the 3 EO strains were within a genetic distance of 0.103.

4.5 Discussion

4.5.1 RAPD analysis of *Trichoderma harzianum*

RAPD analysis of the subset of 12 *T. harzianum* strains revealed that the better biological control strains 7 and 105 grouped with of the poorer strains, being less than 20% dissimilar. This indicates that the RAPD analysis could not completely distinguish between relatively better or poorer biological control strains of *T. harzianum* as determined by the greenhouse tomato petiole bioassay. However, there is a trend towards this type of grouping.

Strains 7 and 29, which were selected as the 2 best performing biological control strains grouped quite differently, being over 75% dissimilar and this could be an indication that their biological control activity towards *B. cinerea* could be due to different mechanisms. If different mechanisms are the basis of the clustering of the *T. harzianum* strains, the better biological control strains 7 and 105, grouping with the poorer strains suggests that there may be little difference separating some good biological control strains and poorer strains.

Different biological control strains of *T. harzianum* can have different biological control capabilities be based on different mechanisms (Harman 2000, Howell 2003). There is potential for the RAPD technique to distinguish between the *T. harzianum* strains based on biological control potential. There is also potential for the RAPD technique to provide a means to distinguish biological control strains based on differential mechanism of biological control between strains. The clustering of strain 7 and 105 with the poorer biological control strains and the distance separating the poorer strains from the better strains poses the question of the nature of the differences in biological control performance with respect to mechanisms. This potential would have to be assessed in further studies which include other assessments based on factors which have been shown to be related to the biological control potential of *Trichoderma* spp. such as chitinase or glucanase activity (Harman 2000; Howell 2003).

It should be possible to develop genetic markers that could be used to screen large numbers of strains of *Trichoderma* spp. for their potential as biological control agents in conjunction with the greenhouse tomato petiole bioassay.

4.5.2 RAPD analysis of *Botrytis cinerea*

RAPD analysis of the 4 subsets of strains of *B. cinerea* demonstrates geographically distinct groups of *B. cinerea* within Alberta. Strains of *B. cinerea* collected from the same location always grouped together. The first subset of *B. cinerea* subjected to RAPD analysis were strains that were all recovered from greenhouse tomato from a number of locations. The resulting phenogram (Fig. 4-2) reveals a strong association between the clusters of strains to the greenhouse from which the strain was collected. The strongest association with geography is to the level of the specific collection location. The LF and BT strains collected from Strathmore did not cluster more closely together because they come from the same area. This is also demonstrated by the 2 sets of WD and VW strains collected from Medicine Hat.

These data clearly demonstrated the presence of geographically distinct groups of *B. cinerea* in Alberta that is associated to specific collection location. Kerssies and Bosker-van Zessen (1997) concluded that the inoculum source in greenhouses in the Netherlands is ubiquitous with a constant flux of conidia into and out of the greenhouse throughout the season. Similar studies in France (Giraud *et al.* 1997; Giraud *et al.* 1999), Spain (Alfonso *et al.*2000) and Chile (Muñoz *et al.* 2002) did not find evidence of geographic distinctness in strains of *B. cinerea*, although there was evidence for host specialization. Giraud *et al.* (1997; 1999) examined *B. cinerea* strains collected from vineyards in the Champagne regions of France. Alfonso *et al.* (2000) studied strains of *B. cinerea* collected from greenhouse vegetable crops in south-eastern Spain. Muñoz *et al.* (2002) worked with field strains of *B. cinerea* collected from a variety of hosts from the Central Valley of Chile.

No reasons for the lack of geographic differentiation in *B. cinerea* strains are presented by Giraud *et al.* (1997; 1999). Alfonso *et al.* (2000) speculated that continuous gene flow must occur between greenhouses preventing differentiation between populations. Alfonso *et al.*(2000) further states that there is an absence of geographic differentiation between strains of *B. cinerea* collected from Italy, the Netherlands, Israel and Spain and speculates that either migration must occur on a larger scale than that of the regions in the study area or populations of *B. cinerea* throughout Europe and the Mediterranean area must have had a recent common origin. Muñoz *et al.* (2002) explains that the Central Valley of Chile has conditions highly conducive to grey mould and that *B. cinerea* is known to have far-migrating spores.

The presence of geographically distinct groups of *B. cinerea* requires that a selection pressure exists to prevent appreciable widespread distribution of *B. cinerea* between locations.

Other studies have reported no geographic differentiation in populations of *B. cinerea* separated by distances of 50 to 530 km (Delcán and Melgarjo 2002; Muñoz *et al.* 2002). The distances separating groups in this study ranged from a few kilometers to 600 km and distance alone is therefore not likely a sole determiner in explaining the development of geographic groups. However, Alberta generally has a climate that is inhospitable to *B. cinerea* for much of the year, and this factor could serve to isolate groups of *B. cinerea*.

Epidemics of *B. cinerea* in Alberta greenhouse crops generally occur early in the crop season from February to May and later in the crop season, September through November. From May to September the greenhouse environment is not favourable to disease development due to the warm temperatures and high ventilation rates bringing in large volumes of dry outside air.

During the periods of epidemics in greenhouses, the outside temperatures are often below freezing during the late winter months and early spring, and again as the early fall progresses towards winter. Spores of *B. cinerea* that are exhausted outside the greenhouse are exposed to a harsh environment, unfavourable to survival and germination with an absence of host plant material, with the exception of the fall period.

The external environment does not favour the widespread dispersal of *B. cinerea* between greenhouses in Alberta during the periods when the internal environment favours disease development. In addition, the summer months are often very dry throughout the province, which may also act to isolate populations of *B. cinerea* that are able to establish in the field.

The presence of vegetative incompatibility groups in *B. cinerea* would act as mechanism of genetic conservation and serve to explain the development of geographically distinct groups within *B. cinerea*. The basis for vegetative incompatibility in fungi is been associated with differences between strains with respect to specific incompatibility loci (Fournier *et al.* 2003). Vegetative compatibility groups are generally considered to be selectively neutral with respect to pathogenicity, virulence and vegetative fitness (Leslie 1993).

An established population in a particular location would less likely to exchange genetic information with introduced strains especially if the strains were from different vegetative compatibility groups. Strains within a vegetative compatibility group are generally more similar than strains from different compatibility groups (Leslie 1993). Strains within a vegetative compatibility group are potentially capable of exchanging genetic information and selection pressure could drive the evolution of distinct characters in sub-populations. It is possible that distinct characters could develop that result in increased vegetative incompatibility, particularly with outside strains.

There were no obvious distinguishing characteristics among the different greenhouses that might provide explanations for the different geographic groups. Tomato greenhouses are generally managed to the same standards and cultivar selection is also generally common throughout the province. The bedding plant greenhouses certainly grow a wider variety of plants and the difference in management from the tomato greenhouses might be a factor in development of distinctive groups.

There are differences in environment across the province and this may contribute to the development of distinctive groups. The light levels are generally considered to be higher in the southeastern corner of the province as compared to the north central region. Outside conditions are hotter and drier during the summer in the south as well.

Differences in the external environment may result in differences in the greenhouse environment even though greenhouse growers manage the environment to obtain the same general environmental conditions.

Geographically distinct groups of *B. cinerea* may result from the selection pressure from subtle differences in the greenhouse environments. Mutation and genetic drift in isolated populations are also mechanisms that should be considered. Sexual reproduction in these groups is another possible source of variation that should be investigated. Diverse phenotypic and genotypic structure in populations of *Phytophthora infestans* from France and Switzerland was suggested to be due to local processes, including selection rather than through long distance migration (Knapova and Gisi 2002). This further suggests that local processes are adequate to result in distinctive population characters.

4.6 Conclusions

This study demonstrated some of the variability within *T. harzianum* and within *B. cinerea* using RAPD analysis. The variability present in *T. harzianum* indicated relationships between RAPD groupings and biological control potential. Further study is required to investigate these relationships and determine whether there is a foundation on which to develop genetic markers for biological control potential that could involve more than one mechanism of control.

RAPD analysis of *B. cinerea* clearly demonstrated the existence of geographic specialization in strains of *B. cinerea* collected in Alberta. The presence of geographically distinct groups of *B. cinerea* presents considerable opportunity to learn more about the genetic structure of this fungus. Geographic specialization in *B. cinerea* has not been reported previously.

4.7 Tables and figures

Table 4-1. Sequences of RAPD primers employed in the genetic analysis of 12 *Trichoderma harzianum* strains demonstrating differences in biological control potential against *B. cinerea* on tomato.

Primer	**Base Sequence**	**No. of Polymorphic Bands Scored**
OPA02	5'-TGCCGAGCTG-3"	22
OPA03	5'-AGTCAGCCAC-3'	22
OPA04	5'-AATCGGGCAC-3'	21
OPB10	5'-CTGCTGGGAC-3'	20
OPC02	5'-GTGAGGCGTC-3'	32
OPC15	5'-GACGGATCAG-3'	13
OPD02	5'-GGACCCAACC-3'	22
OPD20	5'-ACCCGGTCAC-3'	29
OPE15	5'-ACGCACAACC-3'	18
OPE20	5'-AACGGTGACC-3'	20
Total no. of Polymorphic bands		**219**

Table 4-2. Sequences of RAPD primers employed in the genetic analysis of 38 *Botrytis cinerea* strains recovered from tomato from 8 greenhouse locations.

Primer	**Base Sequence**	**No. of Polymorphic Bands Scored**
OPA01	5'-CAGGCCCTTC-3'	10
OPA02	5'-TGCCGAGCTC-3'	16
OPA13	5'-CAGCACCCAC-3'	13
OPA18	5'-CGGTGACCGT-3'	13
OPA20	5'-GTTGCGATCC-3'	9
OPB01	5'-GTTTCGCTCC-3'	9
OPB04	5'-GGACTGGAGT-3'	15
OPB10	5'-CTGCTGGGAC-3'	11
OPD03	5'-GTCGCCGTCA-3'	19
OPD13	5'-GGGGTGACGA-3'	9
Total no. of Polymorphic bands		**124**

Table 4-3. Sequences of RAPD primers employed in the genetic analysis of 50 *Botrytis cinerea* strains recovered from a variety of host plants from 10 greenhouse locations.

Primer	**Base Sequence**	**No. of Polymorphic Bands Scored**
OPA01	5'-CAGGCCCTTC-3'	9
OPA02	5'-TGCCGAGCTC-3'	15
OPA13	5'-CAGCACCCAC-3'	12
OPA18	5'-CGGTGACCGT-3'	13
OPA20	5'-GTTGCGATCC-3'	11
OPB01	5'-GTTTCGCTCC-3'	10
OPB04	5'-GGACTGGAGT-3'	16
OPB10	5'-CTGCTGGGAC-3'	14
OPD03	5'-GTCGCCGTCA-3'	20
OPD13	5'-GGGGTGACGA-3'	10
Total no. of Polymorphic bands		**130**

Table 4-4. Sequences of RAPD primers employed in the genetic analysis of 35 *Botrytis cinerea* strains recovered from a variety of host plants from 31 greenhouse and field locations in Alberta.

Primer	Base Sequence	No. of Polymorphic Bands Scored
OPA01	5'-CAGGCCCTTC-3'	11
OPA02	5'-TGCCGAGCTC-3'	17
OPA13	5'-CAGCACCCAC-3'	14
OPA18	5'-CGGTGACCGT-3'	14
OPA20	5'-GTTGCGATCC-3'	8
OPB01	5'-GTTTCGCTCC-3'	9
OPB04	5'-GGACTGGAGT-3'	17
OPB10	5'-CTGCTGGGAC-3'	16
OPD03	5'-GTCGCCGTCA-3'	19
OPD13	5'-GGGGTGACGA-3'	10
Total no. of Polymorphic bands		**135**

Table 4-5. Sequences of RAPD primers employed in the genetic analysis of 35 *Botrytis cinerea* strains recovered from a number of host plant species and assessed for virulence against tomato, cucumber, geranium, and petunia in stem piece bioassays.

Primer	**Base Sequence**	**No. of Polymorphic Bands Scored**
OPA01	5'-CAGGCCCTTC-3'	12
OPA02	5'-TGCCGAGCTC-3'	17
OPA13	5'-CAGCACCCAC-3'	15
OPA18	5'-CGGTGACCGT-3'	22
OPA20	5'-GTTGCGATCC-3'	8
OPB01	5'-GTTTCGCTCC-3'	9
OPB04	5'-GGACTGGAGT-3'	17
OPB10	5'-CTGCTGGGAC-3'	15
OPD03	5'-GTCGCCGTCA-3'	20
OPD13	5'-GGGGTGACGA-3'	10
Total no. of Polymorphic bands		**145**

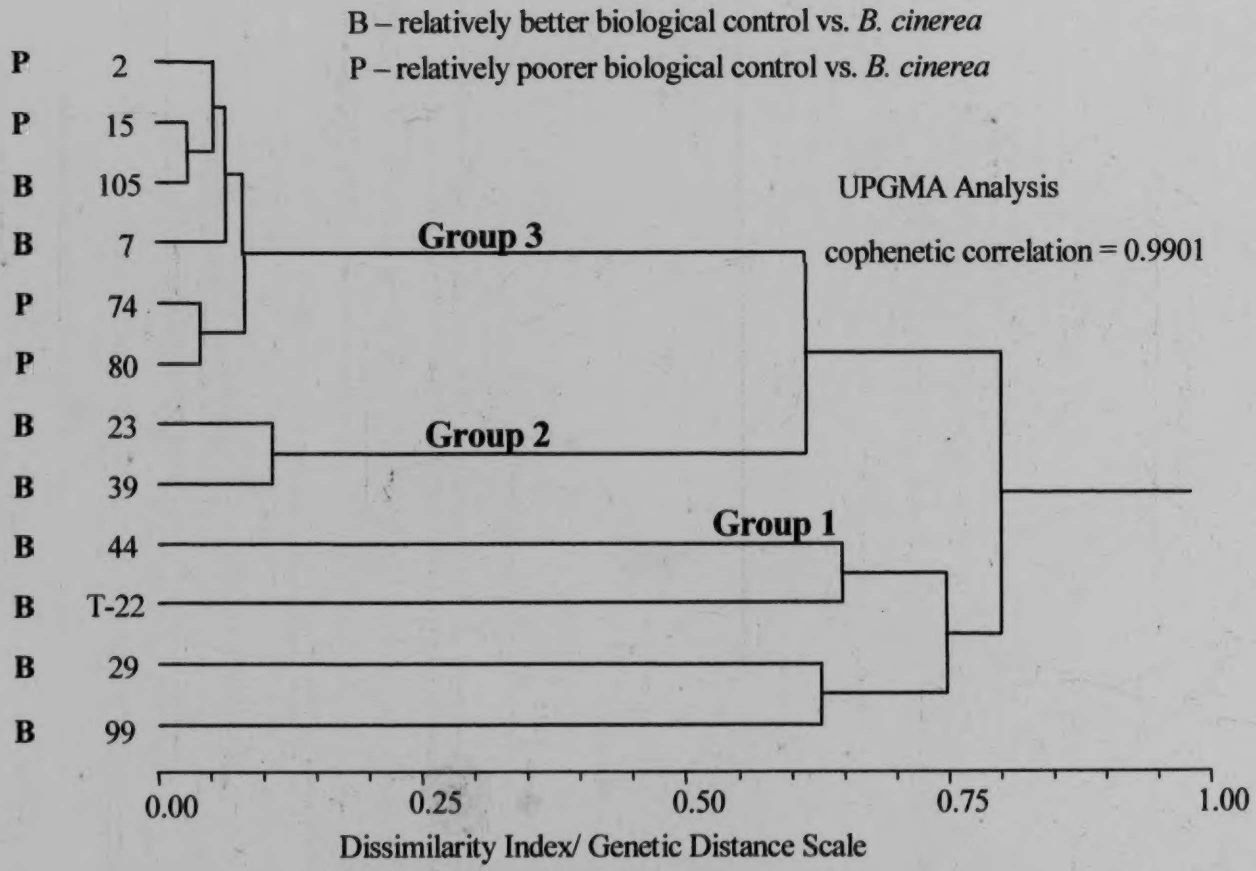

Figure 4-1. Genetic relationships among 12 *Trichoderma harzianum* strains exhibiting differences in biological control efficacy against *Botrytis cinerea* on tomato. T-22 is the commercial biological control strain sold as RootShield®

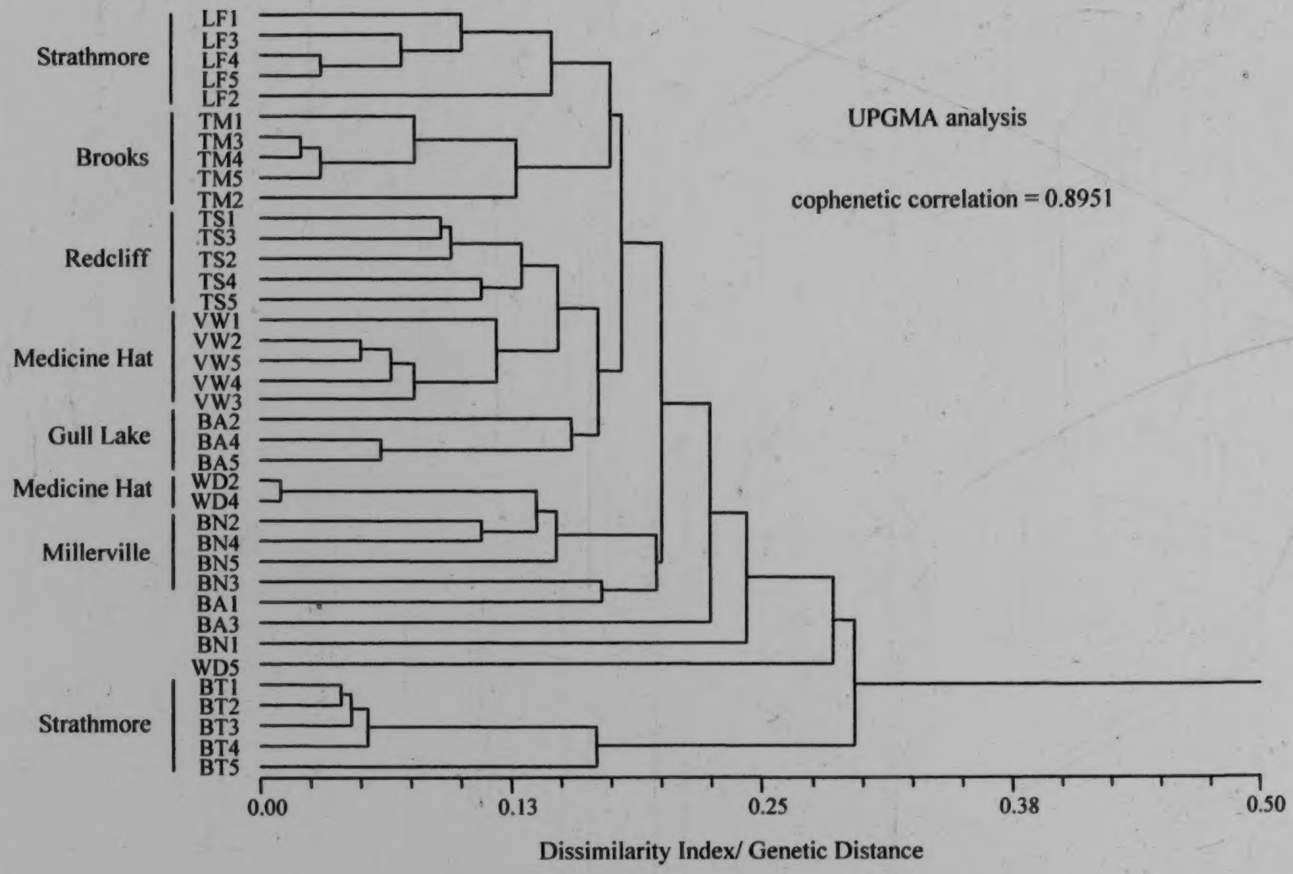

Figure 4-2. Genetic relationships among the 38 *Botrytis cinerea* strains collected from tomato in 8 greenhouse locations in Alberta

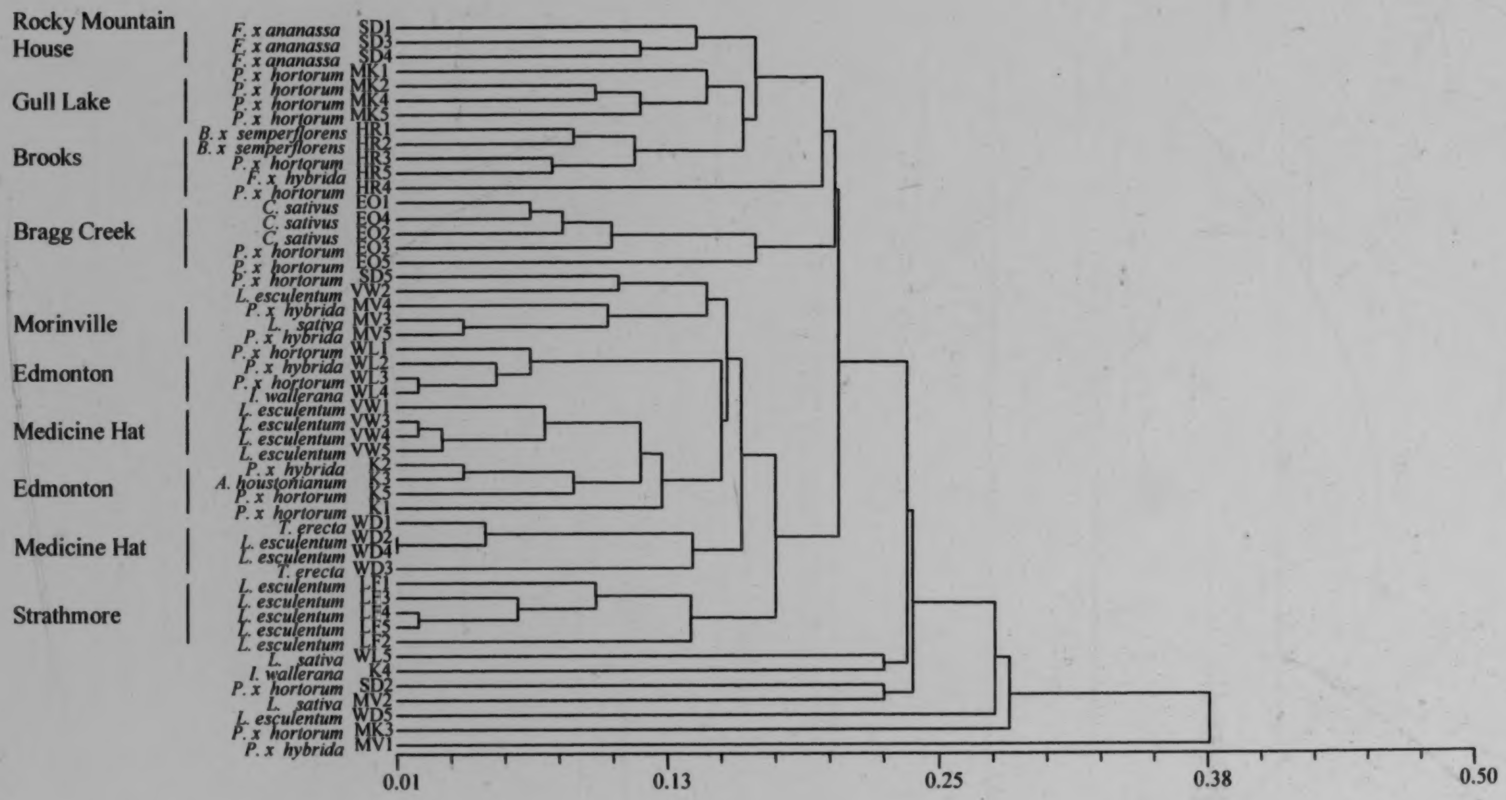
Rocky Mountain House
Gull Lake
Brooks
Bragg Creek
Morinville
Edmonton
Medicine Hat
Edmonton
Medicine Hat
Strathmore
F. x ananassa SD1
F. x ananassa SD3
F. x ananassa SD4
P. x hortorum MK1
P. x hortorum MK2
P. x hortorum MK4
P. x hortorum MK5
B. x semperflorens HR1
B. x semperflorens HR2
P. x hortorum HR3
F. x hybrida HR5
P. x hortorum HR4
C. sativus EO1
C. sativus EO4
C. sativus EO2
P. x hortorum EO3
P. x hortorum EO5
P. x hortorum SD5
L. esculentum VW2
P. x hybrida MV4
L. sativa MV3
P. x hybrida MV5
P. x hortorum WL1
P. x hybrida WL2
P. x hortorum WL3
I. wallerana WL4
L. esculentum VW1
L. esculentum VW3
L. esculentum VW4
L. esculentum VW5
P. x hybrida K2
A. houstonianum K3
P. x hortorum K5
P. x hortorum K1
T. erecta WD1
L. esculentum WD2
L. esculentum WD4
T. erecta WD3
L. esculentum LF1
L. esculentum LF3
L. esculentum LF4
L. esculentum LF5
L. esculentum LF2
L. sativa WL5
I. wallerana K4
P. x hortorum SD2
L. sativa MV2
L. esculentum WD5
P. x hortorum MK3
P. x hybrida MV1
0.01
0.13
0.25
0.38
0.50

Figure 4-3. Genetic relationships among the 50 *Botrytis cinerea* strains collected from tomato in 16 greenhouse locations in Alberta

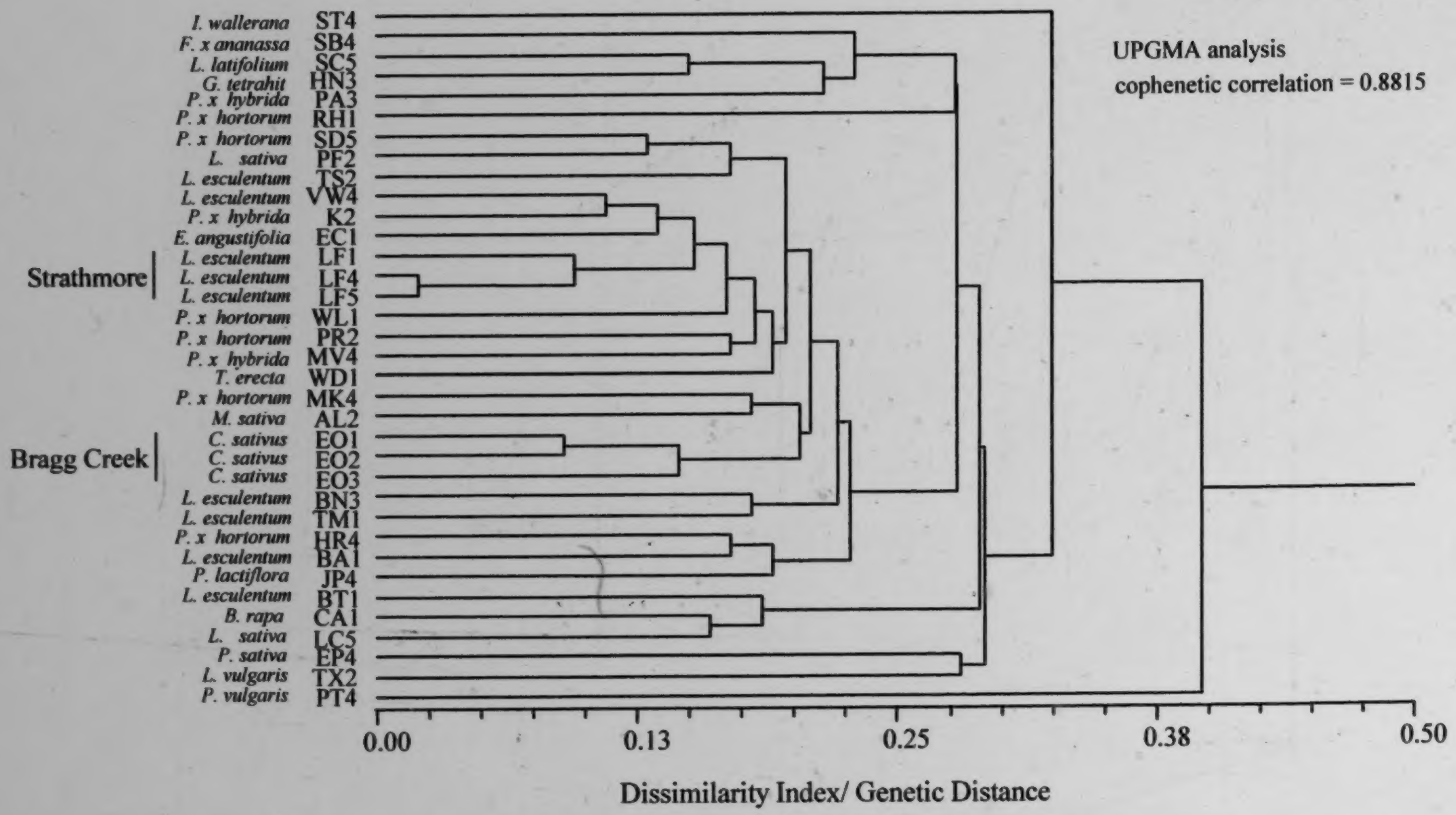

Figure 4-4. Genetic relationships among 35 Botrytis cinerea strains colleced from a number of host plant species from 31 greenhouse and field locations in Alberta

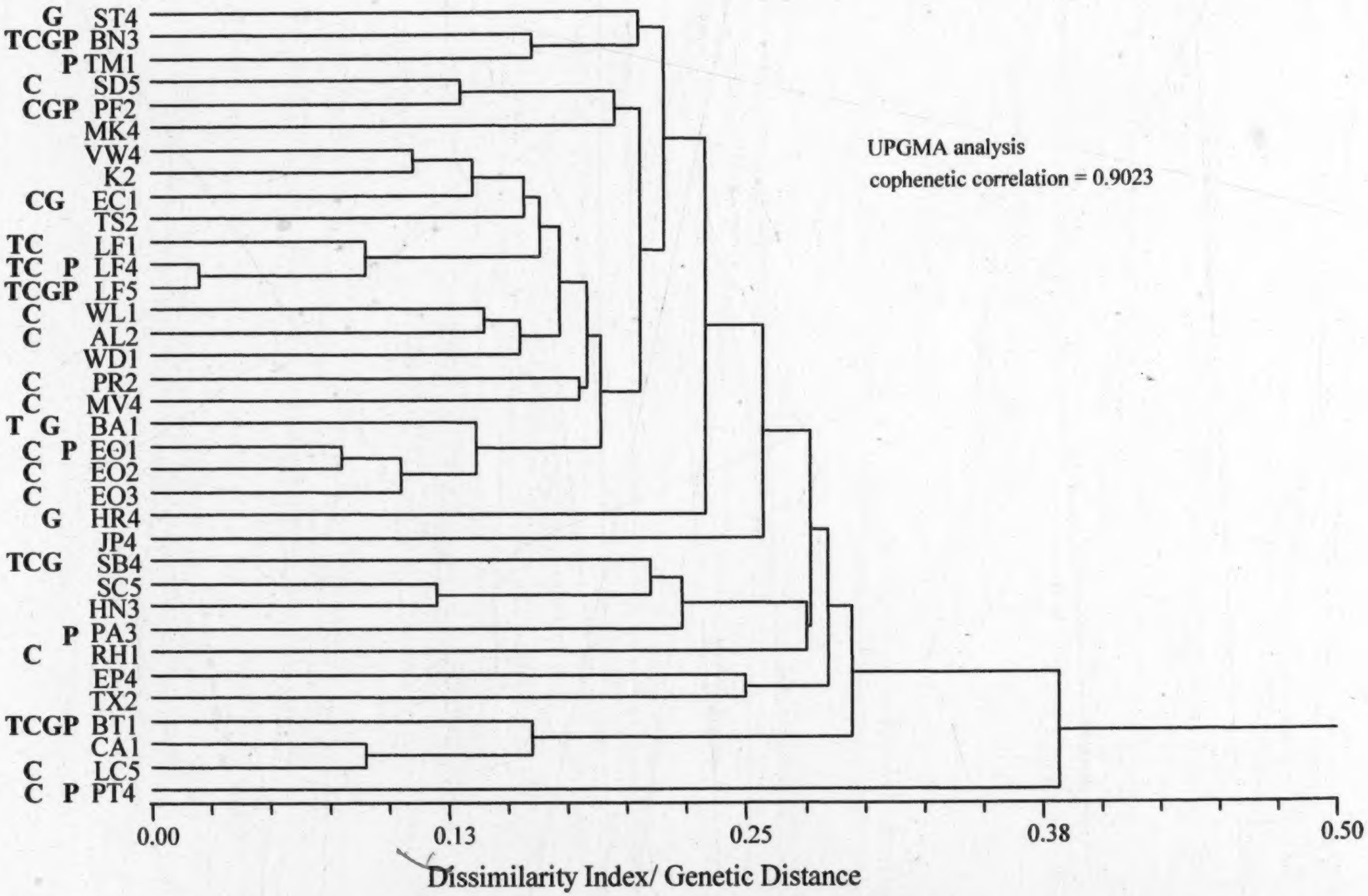

Figure 4-5. Genetic relationships among the 35 *Botrytis cinerea* strains collected from a number of host species and assessed for virulence against tomato (**T**), cucumber (**C**), geranium (**G**), and petunia (**P**) in stem piece bioassays.

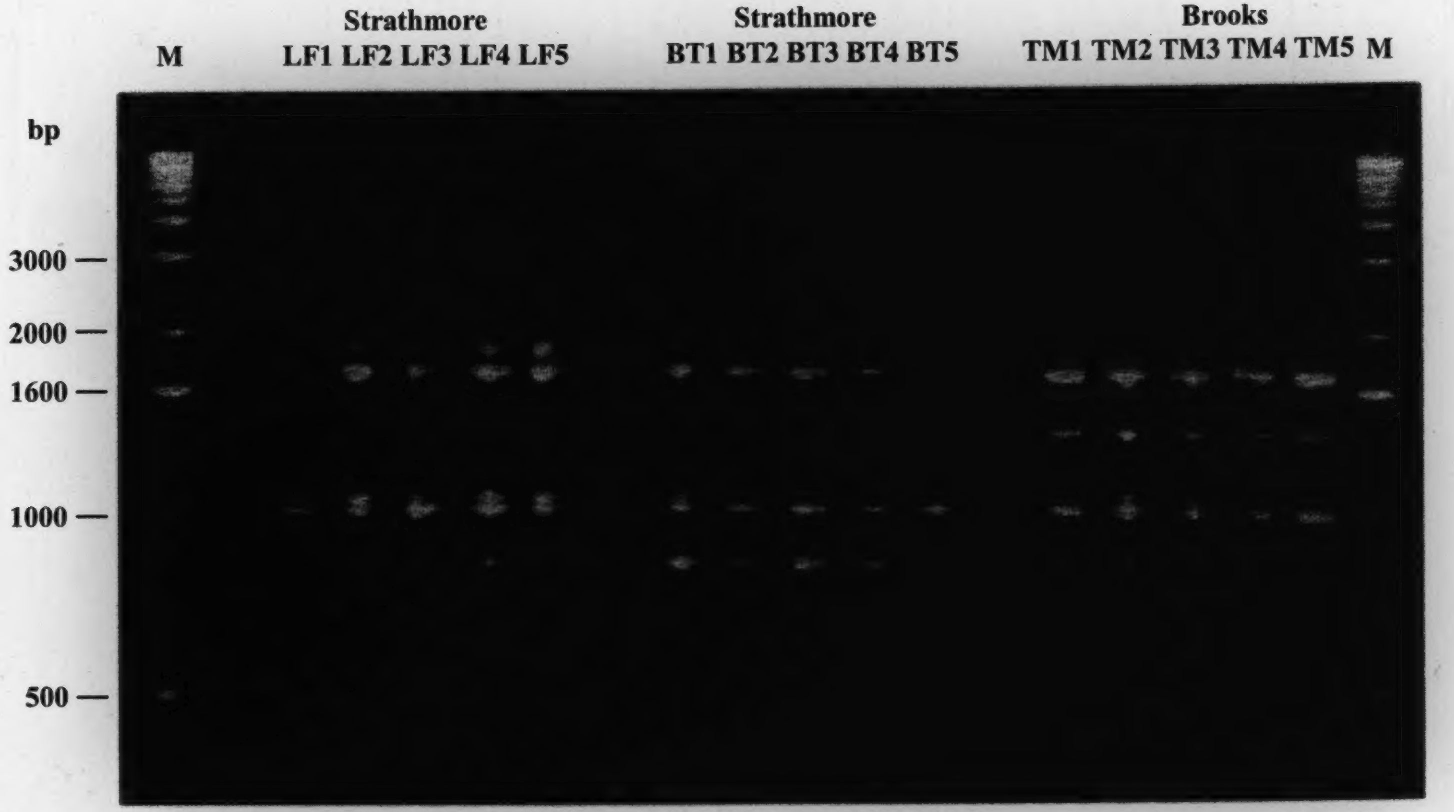
Strathmore
Strathmore
Brooks
M
LF1 LF2 LF3 LF4 LF5
BT1 BT2 BT3 BT4 BT5
TM1 TM2 TM3 TM4 TM5
M
bp
3000
2000
1600
1000
500

Figure 4-6. RAPD patterns of 15 *Boytris cinerea* strains collected from tomato from 3 greenhouse locations. Patterns obtained by amplification of total DNA using 10-mer primer OPB01 (5'-GTTTCGCTCC-3'). DNA ladder (1kb) indicated as M.

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Chapter 5

5.1 General Discussion

Agriculture in developed countries undergoes continuous change in order to maintain demand for product in a market where consumer decisions are increasingly based on ethical considerations of production practices. The world's population is expected to increase by 45 per cent over the next 30 years, putting considerable pressure on global agriculture to provide enough food (Mannion 1998). Agriculture in developed countries is also under pressure to meet this need in a manner that is more sustainable. Sustainable agriculture has been defined as the management of agricultural practice to satisfy human needs while maintaining or enhancing the quality of the environment and conserving natural resources (Conway 1996). One of the major objectives of sustainable agricultural systems is to reduce inputs into crop production (Conway 1996). The adoption of sustainable practices by producers could result in the reduction or elimination of pesticides and the development and use of biological controls will become more important (Conway 1996).

Biological control is an important component of sustainable agriculture because it provides an alternative to the use of chemicals. In the classical concept of biological control, organisms are released into the environment and become self-perpetuating while providing acceptable control from year to year (Conway 1996). Conway recognized that industry may not be interested in developing biological control products that maintain populations in the environment. Another scenario for the use of biological control agents is for the repeated applications of these agents. This inundative approach is similar to the repeated application of chemical control agents, and does not represent a reduced input into the production system. It does establish a more favourable profit picture that would provide support to research and development of biological control agents.

A sustainable agriculture that is able to increase food production to meet the requirements of the next 30 years will likely require increased inputs, especially if the world's remaining natural ecosystems are to be preserved. The nature of these inputs and the production systems in which they are managed will also likely have to change to reduce the negative impacts of agricultural production on the environment. The application of new information to production systems can result in more sophisticated approaches to management, that may result in reduced use of chemicals, but also represent an increased intellectual input.

Greenhouse crop production has the capacity to increase crop production per unit area substantially over comparable field production systems. Greenhouse crop production will likely play a more prominent role in meeting the world's food requirements. Greenhouse crop production requires higher inputs to provide the environment required to optimize crop production. The ability to modify the environment also allows for management of the environment to optimize performance of biological control agents.

Biological control agents are desirable and are used successfully under greenhouse crop production. Biological control of insect pests in greenhouses is an established practice. Biological control agents are a natural resource offering considerable potential for disease control, and much of this potential remains untapped (Butt and Copping 2000). Biological control agents are required to manage greenhouse crop disease such as those caused by *B. cinerea*. There are no such biological control agents currently registered in Canada. The overall objective of this thesis research was to develop a biological control for *B. cinerea* in greenhouse tomato using a strain of *Trichoderma* sp. from Alberta.

Considerable effort and expense is required in the commercialization of biological control products (Harman 2000) and ensuring that biological control candidates are selected using a selection process that include a highly virulent strain of the pathogen is a prudent practice, if not a necessity. Biological control agents selected for further development based on promising performance against less virulent strains of the pathogen may not stand up as well under field conditions. Failure to obtain a registration for a biological control product represents a significant loss for the company that developed the product. More importantly is that the registration of biological control products that do not perform well in the field can damage the reputation of biological control agents as a whole. The use of highly virulent strains of the target pathogen early in the selection process should improve the probability of developing a more effective biological control product. This study provides a method to select a highly virulent strain of *B. cinerea*, for use as the target strain of this pathogen, in the screening of potential biological control agents. A more rigorous selection process can be established if a highly virulent strain of the target pathogen is identified and selected for use in the process.

5.1.1 Virulence of *Boytris cinerea*

The stem-piece bioassays provided a means to differentiate the strains of *B. cinerea* based on virulence. The bioassays demonstrated that the virulence of some strains were high on all hosts while virulence of other strains varied across the range of the 4 host plant species. There was no clear evidence of host specificity although strains recovered from a plant species were generally pathogenic on that plant species in the bioassays. Some strains were highly virulent on one or two of the host plant species. Variability in virulence on the host plants was evident among strains collected from the same location. These results support the generally accepted view that *B. cinerea* is not host specific and expresses a high degree of variability in virulence with the ability to parasitize a wide range of hosts. Strains of *B. cinerea* demonstrate some host specialization yet populations of this pathogen are likely to contain and express the genetic capability to cause disease on a wide host range.

The diversity in virulence of the *B. cinerea* strains indicate that many genes are involved in pathogenesis. Research with single *B. cinerea* genes involved in pathogenicity and virulence has demonstrated that disruption of these individual genes may reduce pathogenicity and virulence of the fungus, but does not prevent disease development. It is possible that all pathogenicity genes present in *B. cinerea* could be present in each strain, and that it is the expression of genes by different strains and in different hosts that varies. Wubben *et al.* (1999) characterized the 6 endo-polygalacturonase genes from *B. cinerea* and found that each member of the gene family was present in all the *B. cinerea* strains tested as well as being present in all of other strains of *Botrytis* spp. examined. Differential expression of these genes can vary with host (ten Have *et al.* 2001). It has been demonstrated that 6 endo-polygalactruonase genes are present in *B. cinerea*. The requirement for a number of isozymes could be due to differences in substrate and environment and that the functional overlap of a number of genes would then not be a case of redundancy. In some cases single *B. cinerea* genes are required for pathogenesis (Zheng *et al.* 2000).

Differential expression of these genes would allow for variability in pathogenesis and virulence between strains of *B. cinerea* within a population. It would explain the presence of strains which are virulent on a number of hosts, strains that display apparent host specialization, and strains that are weakly virulent on a number of hosts.

Mutation is the primary cause of heritable variation in fungi and 2 mechanisms are believed to govern this variation in *Botryis* spp. and in its perfect state *Botryotinia* spp., these mechanisms are sexual recombination and heterokaryosis. Heterokaryosis is the generally accepted mechanism responsible for the genetic variability in *B. cinerea* (Delcán and Melgarejo 2002), however it is not known if heterokaryosis occurs in nature. The degree to which sexual recombination occurs in nature is also not well understood.

The evidence supporting that a vegetative incompatibility system may be operating in *B. cinerea* (Fournier *et al.* 2003) reduces the expected degree of heterokaryosis. This also increases speculation that the extensive diversity in *B. cinerea* may not be due to heterokaryosis. A system of vegetative incompatibility in *B. cinerea* or an absence of heterokaryosis in nature, or heterokaryosis that results only in transient heterokaryons, all place restrictions on the development of genetic variability.

Alternative proposed mechanisms such as interstrain gene transfer may explain the degree of diversity that exists in *B. cinerea*. Interstrain gene transfer proposes that only small pieces of DNA move from 1 genome to the other following anastomosis and these pieces are incorporated into the genome of the recipient strain (Harman *et al.* 1998). Interstain gene transfer is a variation on the theme of the heterokaryosis concept but does not require the formation of heterokaryons and could operate following brief anastomosis events.

The multinucleate condition of conidia and hyphal cells of *B. cinerea* also provides an explanation for a high degree of diversity. Differential gene expression could occur among the various nuclei in cells in response to environmental conditions, and could give rise to many permutations of expression even within a population. If intergene transfer exists it would allow for interstrain exchange of DNA, even if only at a lesser rate than is possible under heterokaryosis.

This study is the first to characterize the virulence of *B. cinerea* strains, collected from a wide variety of diseased host plant material, against tomato and other host plants as the basis for selecting a highly virulent strain for use in evaluating potential biological control agents. The results of this study demonstrate variability in pathogenicity and virulence among strains of *B. cinerea* collected from the same or different plant hosts at 1 location as well as between strains collected from different hosts and different locations.

This study demonstrates that *B. cinerea* strains recovered from diseased plant material in the field will likely be pathogenic, although the virulence on the intended host plant cannot be predicted. Individual strains from the same population can vary in virulence and that populations from different locations also vary in virulence. The capability to cause disease does not provide any indication of virulence on the host plant species of interest. A screening process is required to characterize pathogenicity and virulence of field strains.

5.1.2 Model system for commercial greenhouse tomato production

The development of a successful biological control agent is dependent on the selection process. An important consideration of the selection process is the environment in which the biological control agent is expected to perform. This includes the intended crop and physical environment in which the crop is grown, as well as the strain of the pathogen used.

Mass screening of potential biological control agents for use in greenhouse crops is best conducted in research greenhouses that attain commercial levels of production. The greenhouse tomato petiole bioassay was an intensive assay as it required large facilities and capabilities to grow a high yielding greenhouse tomato crop. However, once the greenhouse production system is established it represents an important, practical component of a development program for biological control agents. This production capability would increase the effectiveness and success of programs having the means for considerable in-house commercial screening, in advance of large-scale commercial evaluations. Such greenhouses should be integral components of research programs developing biological control agents for greenhouse crops.

Assays that involve live plants in a high yielding crop production environment more closely simulate the actual commercial environment and improves the field relevance and rigour of the assay. High yielding tomato plants in high-density crop stands are under considerable stress to set and fill fruit and may also be more susceptible to infection by *B. cinerea* than vegetative tomato plants under low density or low yielding situations.

This study provided a practical approach to develop a greenhouse tomato model system that is representative of a commercial tomato greenhouse. This system can obtain high yield of commercial quality tomatoes and can be used for the mass screening and development of strains of *T. harzianum* as biological control agents for *B. cinerea* in tomato. This greenhouse tomato production model system also presents an excellent opportunity for wider application of studies in greenhouse tomato crops.

5.1.3 *Trichoderma harzianum* as a biological control agent for *Botrytis cinerea*

Strains of *Trichoderma* spp. were isolated from soil and compost samples and *T. harzianum* accounted for the majority of the collected strains. *Trichoderma* spp. have a worldwide distribution and are frequently dominant components of soil microflora in a variety of habitats. There is a great degree of diversity in the genus and new species continue to be discovered. The strains of *T. rossicum* recovered from locations in Brooks and Edmonton, during the course of this study, represent the first Canadian record of this new species.

Trichoderma harzianum is one of the most common species within the genus and is commonly known as a biological control agent against a wide variety of plant pathogenic fungi (Gams and Meyer 1998). The majority of the strains collected in this study were *T. harzianum* strains with 66 of the 109 strains.

None of the assays used in this study were based on any specific indicators of the mode of action of *Trichoderma* spp., such as differential production of enzymes such as chitinases or glucanases. Even when using these specific indicators to select the most promising biological control strains, commercialization assessments would still have to include trials in the intended environment and the target host crop.

Competition is reported to be the primary mode of action of Trichodex ® (*T. harzianum* strain T-39) against *B. cinerea* (Elad and Kapat 1999). Strain T-39 does not act through volatile or non-volatile antibiotics. No mycoparasitism has been observed in the interaction between *T. harzianum* strain T-39 and *B. cinerea*. However, Elad and Kapat (1999) reported that protease activity is probably involved in the biological control of *B. cinerea* by *T. harzianum* strain T-39.

Of the three assays used to assess the biological control potential of *Trichoderma* spp. against *B. cinerea*, the greenhouse tomato petiole bioassay gave the widest discrimination in biological control performance among the *Trichoderma* spp. strains. The assay is particularly useful because it is focused on the performance of *Trichoderma* spp. strains against *B. cinerea* in the same situation for which the biological control was being developed. It was the most direct assessment of how well the biological control organism can be expected to perform.

It is an assay that does not require an understanding of the mechanisms of control, but it does establish an environment for subsequent study of the biological control mechanisms involved. Studies investigating the mechanisms of control of biological control agents should be conducted in the field as control mechanisms can vary based on the environment.

Disease control practices in commercial greenhouses are often integrated and growers will attempt to modify the environment to prevent optimum conditions for disease development and use fungicides to protect from infection. In a situation where *B. cinerea* is a problem, any action to limit direct wounding of tomato stems reduces the likelihood of stem infections. Greenhouse tomato crops require routine harvesting and pruning that wound the plant and wounds caused on the petiole may have an effect on the likelihood of stem infection and development of stem cankers. Removal of leaves directly from the node creates wounds directly on the stem. Leaf pruning to leave 3 centimeters of petiole stub still attached to the stem places the wound a distance away from the stem.

Creating a wound a few centimeters away from the stem provides the opportunity to intervene with petiole infections before they reach the stem and develop into stem cankers. Tomato plants are pruned twice a week and petiole infections can be identified and treated with a fungicide before the canker reaches the stem. Treatment involves excising the infected portion of the petiole and an application of fungicide to protect the wound. The tomato petiole bioassay simulates this type of situation where the biological control agents can be evaluated to protect wounds from colonization by *B. cinerea* and provide acceptable alternatives for fungicide use.

Trichoderma harzianum strains 7 and 29 were as effective as fungicides in controlling *B. cinerea* (LF5) in greenhouse tomato as determined, in this study by, using the greenhouse petiole bioassay. Both the biological control agent and fungicide treatments reduced the length of petiole colonization to a level that would provide an opportunity for commercial greenhouse growers to excise infected tissue and treat with a biological control agent in order to protect the wound from infection and prevent the lesion from progressing into the main stem.

Whole-crop pruning operations should be completed within 8 hours. This makes it possible for a biological control application to be made before *B. cinerea* establishes new infections. The results of this study demonstrates that effective control of *B. cinerea* can be obtained by applying *T. harzianum* strains 7 and 29 at periods of time up to 3 hours after inoculation by *B. cinerea*.

The tomato petiole assay used in the selection of *T. harzianum* strains 7 and 29 as biological control agents integrates the use of these agents with cultural methods to prevent petiole infections from reaching the main stem. This approach will require repeated application of the biological control strains in order to place *T. harzianum* in contact with *B. cinerea* on wounds.

5.1.4 Molecular characterization of *Trichoderma harzianum* and *Botrytis cinerea*

This study demonstrated some of the variability present within *T. harzianum* as well as within *B. cinerea* using RAPD analysis. The variability present in a subset of 12 *T. harzianum* strains indicated relationships between RAPD groupings and biological control potential. All of the "poorer" biological control strains grouped together with the majority of the "better" biological control strains grouping separately with more than 75% dissimilarity to the poorer strains.

Strains 7 and 105 were better biological control strains that grouped with of the poorer strains indicating that the RAPD analysis could not completely distinguish between relatively better or poorer biological control strains of *T. harzianum* as determined by the greenhouse tomato petiole bioassay. Strains 7 and 29, which were the 2 best performing biological control strains grouped quite differently, being over 75% dissimilar and this could be an indication that their biological control activity agianst *B. cinerea* could be due to different mechanisms. If different mechanisms are the basis of the clustering of the *T. harzianum* strains, the better biological control strains 7 and 105, grouping with the poorer strains suggests that there may be very little difference separating some good biological control strains and some poorer strains.

Different biological control strains of *T. harzianum* can have different biological control capabilities that are based on different mechanisms (Howell 2003). There is potential for the RAPD technique to distinguish between the *T. harzianum* strains based on biological control potential. There is also potential for the RAPD technique to provide a means to distinguish biological control strains based on differential mechanisms of biological control between strains. The clustering of strain 7 and 105 with the poorer biological control strains and the distance separating the poorer strains from the better strains poses the question of the nature of the differences in biological control performance with respect to mechanisms.

RAPD analysis of the 4 subsets of strains of *B. cinerea* demonstrates geographically distinct populations of *B. cinerea* within Alberta. Strains of *B. cinerea* collected from the same location always grouped together. The strongest association with geography is to the level of the specific collection location.

This grouping based on collection location was present even when the strains were collected from different host plant species at the same location. Strains collected from the same host plant species from different locations did not tend to group together. This suggests general mechanisms of pathogenesis across a wide variety of host plants and is consistent with what would be expected in non-specific necrotrophic plant pathogens.

The third subset of *B. cinerea* strains subjected to RAPD analysis removed much of the location effect from the analysis as each collection location was represented by 1 strain. Strains collected from both the greenhouse and field were included and there were no clusters that correspond to the host plant from which the strains were collected with the exception of the 3 LF tomato strains that clustered together and the 3 EO cucumber strains that also clustered together

The fourth subset of *B. cinerea* strains were collected from a number of host plant species from the greenhouse and field, and assessed for virulence against a number of host plant species. The resulting phenogram did not indicate any clustering on the basis of virulence on a specific host or group of hosts. The only clusters that could be clearly identified were associated with geography and included the 3 LF strains and the 3 EO strains that formed 2 distinct groups.

These studies clearly demonstrated the presence of geographically distinct groups of *B. cinerea* in Alberta that is associated to specific collection location.

There were no obvious distinguishing characteristics among the different greenhouses. There are differences in environment across the province and this may contribute to the development of distinctive groups. Differences in the external environment may result in differences in the greenhouse environment even though greenhouse growers manage the environment to obtain the same general environmental conditions. Geographically distinct groups of *B. cinerea* may result from the selection pressure due to subtle differences in the greenhouse environments. Diverse phenotypic and genotypic structure in populations of *Phytophthora infestans* from France and Switzerland was suggested to be due to local processes, including selection rather than through long distance migration (Knapova and Gisi 2002). This further suggests that local processes are adequate to result in distinctive population characters.

This study demonstrated some of the variability within *T. harzianum* and within *B. cinerea* using RAPD analysis. The variability present in *T. harzianum* indicated the relationships between RAPD groupings and biological control potential. Further studies are required to investigate these possible relationships and determine whether there is a foundation on which genetic markers can be developed for biological control potential that could involve more than one mechanism of control.

RAPD analysis of *B. cinerea* clearly demonstrated the existence of geographic specialization in strains of *B. cinerea* collected in Alberta. The presence of geographically distinct groups of *B. cinerea*, presents considerable opportunity to learn more about the genetic structure of this fungus. This is the first report of geographic specialization in *B. cinerea*.

5.2 Conclusions

1. The RAPD-PCR technique revealed geographically distinct groups of *B. cinerea* in Alberta.

2. Considerable diversity in virulence and pathogenicity exists within strains of *B. cinerea*.

3. Considerable diversity in biological control potential against *B. cinerea* exists within Alberta strains of *Trichoderma* spp. RAPD-PCR analysis showed promise for distinguishing strains of *T. harzianum* on the basis of biological control potential.

4. The strains of *T. rossicum* collected in this study represent the first Canadian record of this species.

5. Two strains of *T. harzianum* were selected as biological control agents for *B. cinerea* in greenhouse tomato. The performance of these strains

was comparable to that of selected fungicides as assessed by using the greenhouse tomato petiole bioassay.

6. A practical model system was developed to grow greenhouse tomatoes to commercial standards for use as the environment in which to develop biological control agents for the management of *B. cinerea*.

The 2 strains of *T. harzianum* selected in this study represent effective, biological control agents for the management of *B. cinerea* in commericial greenhouse tomato crops. The greenhouse tomato model system provides a practical tool for the study of *in vivo* mechanisms of biological control that has specific relevance to the commercial environment of application.

5.3 Future Research

1. **Investigation of *in vivo* mechanisms of biological control activity of *Trichoderma harzianum*.**

The greenhouse tomato production model system and the tomato petiole bioassay offer an opportunity to investigate the mechanisms of biological control of *T. harzianum* in the field environment.

2. **Identification of genetic markers in *Trichoderma harzianium* for mechanisms of biological control.**

The RAPD-PCR technique shows promise for the identification of genetic markers for mechanisms of biological control present in biological control agents. Genetic markers for biological control potential could be developed for a number of mechanisms of control. Markers could be incorporated into assays identifying promising biological control strains and could form the basis for biological control strain improvement.

3. **Investigation of the genetic basis for the level of diversity present in *Botrytis cinerea* from different geographic populations.**

The existence of geographically distinct populations of *B. cinerea* should allow for a more complete understanding of the underlying mechanisms responsible for genetic variation of *B. cinerea* and may lead to the development of novel control strategies. It is important to determine the level of that sexual recombination occurs in the field. This information will allow for a more complete understanding of *B. fuckeliana* (*B. cinerea*).

4. Investigate combinations of biological control agents.

Improvement of biological control efficacy in the field could involve more than one strain of *T. harzianum* for the control of *B. cinerea* in greenhouse tomato. Combinations could also be developed that provide integrated control for more than one disease. It may be possible to develop biological control products that integrated disease control agents with insect control agents. For example, a mixture of *T. harzianum* and the entomopathogenic fungus *Beauveria bassiana* (Bals.-Criv.) Vuill.

5. Investigate other methods of application of biological control agents in the greenhouse.

In order to increase the dissemination and distribution of *T. harzianum* in the tomato crop other methods of application should be investigated and integrated into control programs. The use of bumble bees (*Bombus impatiens* Cresson) has been shown to be a reliable and effective vector for the delivery of *T. harzianum* to strawberry flowers for the control of *B. cinerea* in strawberry crops (Kovach *et al.* 2000). Bumble bees are also used as pollinators in greenhouse tomato crops and represent an opportunity to improve biological control of *B. cinerea* in tomato.

6. Development of production model systems for other greenhouse crops.

The development of production model systems for other greenhouse crops should allow for improved selection and development of biological control agents for these crops as well as biological control products that have broader application to a number of crops.

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Appendix 1

Development of a model system for greenhouse tomato production to commercial standards[1].

Introduction

Greenhouse evaluations have been undertaken to evaluate the biological control activity of promising *T. harzianum* biological control candidates for the control of *B. cinerea* in commercial tomato crops. Greenhouse trials are conducted in research greenhouses on tomato plants under near-commercial conditions or in commercial greenhouse tomato crops (Dik and Elad 1999; Utkhede and Mathur 2002).

Trials conducted on tomatoes under near-commercial conditions may not adequately represent a commercial greenhouse tomato crop as the planting density and yields can be considerably less than commercial crops. Utkhede and Mathur (2002) conducted greenhouse trials in a tomato crop that yielded approximately a quarter of what is obtained in commercial crops. Studies conducted in commercial greenhouses represent the best test of a biological control agent but are best left for the final assessments of very promising biological control agents. Mass screening of potential biological control agents can demand a considerable amount of resources and planning and probably represent too much of an imposition on commercial greenhouses.

Mass screening of potential biological control agents is best conducted in research greenhouses that attain commercial levels of production. Such greenhouses would be integral components of research programs developing biological control agents. This production capability would increase the effectiveness and success of programs having the means for considerable in-house commercial screening, prior to the large-scale commercial evaluations of the most effective biological control candidates.

A greenhouse is a controllable dynamic system (Seginer *et al.* 1996), managed for intensive production of high quality, fresh market produce. There are a number of variables that greenhouse growers have to manage in order to obtain maximum sustainable production from their crops. These variables include; air temperature, root zone temperature, vapour pressure deficit, fertilizer feed, carbon dioxide enrichment, selection of growing media, and plant maintenance. The task of managing these inter-related variables is sometimes considered an art (Seginer *et al.* 1996). The strategy is to optimize these variables to obtain maximum performance from the crop over the entire production season.

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[1] A version of this chapter has been published: Calpas, J.T. 2003. Commercial greenhouse tomato production in Alberta. Alberta Agriculture and Rural Development. Edmonton

Average commercial yields of greenhouse tomato in Alberta are in the range of 45 to 50 kg m^{-2} with top growers able to reach 60 kg m^{-2} in some years.

The objective of this study was;

1. To develop a production model system to grow greenhouse tomatoes according to commercial standards to attain commercial levels of production for use as the environment to screen and develop a biological control agent for the biological control of *B. cinerea* in greenhouse tomato.

This study was conducted to develop a commercial greenhouse tomato model system as an integral component of a biological control research and development program. There are currently no reports of biological control programs having this capability.

Materials and methods

Two tomato production yield trials were conducted during the 1998-1999 and 1999-2000 greenhouse tomato commercial crop years. Production techniques followed were based on techniques used by Alberta commercial tomato growers. The recommendations in the Greenhouse Vegetable Production Guide for Commercial Growers (British Columbia Ministry of Agriculture, Fisheries and Food) (Portree *et al.* 1996) were also used as an approximate guide. Greenhouse crop production strategies developed for one area may not be optimal for other areas (Jones *et al.* 1989). As the Alberta growing conditions are significantly different from the conditions in British

Columbia, with higher light levels and lower relative humidity, adaptations of the recommendations of the British Columbia guide were carefully considered.

During the 1998-1999 crop year, plants from 2 separate seed lots of tomato, cultivar Rapsodie (Novartis Seeds Inc.), were grown. Four replicate treatments of 8 plants from each of the 2 Rapsodie seed lots were arranged in a randomized design in a 120 m^2 research greenhouse at the Crop Diversification Centre South at Brooks Alberta. Each treatment occupied an area of 4 m^2. Seeding was done in a 120 m^2 nursery greenhouse on November 15, 1998. The seedlings were transplanted into the production greenhouse at 5 weeks of age, on December 20. Yield data was collected and included grade of fruit harvested.

In 1999-2000 plants from 3 tomato cultivars, Rapsodie (Novartis Seeds Inc.), Quest (De Ruiter Seeds) and Trust (De Ruiter Seeds) were seeded, transplanted and grown according to the same schedule as followed in 1998-1999. Four replicate treatments of 8 plants from each cultivar were arranged in a randomized design in the 120 m^2 production greenhouse. Each treatment occupied an area of 4 m^2. The 2 extra outside rows surrounding the crop were kept as guard rows in both years of the trial. Yield data was collected for and included grade of fruit harvested.

The plants were watered with a complete nutrient solution consisting of: $Ca(NO_3)_2$, 950 g; KNO_3, 500 g; KH_2PO_4, 140 g; KSO_4, 240 g; $MgSO_4$, 500 g; micronutrients; iron chelate (13.2% iron), 20 g; manganese chelate (13% manganese), 0.1g; copper chelate (14% copper), 0.01 g ; sodium molybdate (46% molybdenum), 0.1 g; zinc chelate (14% zinc); 1 g, and borax (15% boron), 3 g, per 1000L of water. The nutrient solution was adjusted to pH 5.8 by the addition of 100 mL of H_2PO_4, the electrical conductivity (EC) of the solution was 2500 $\mu s\ cm^{-2}$.

Seeds were sown directly into 2 cm x 2 cm x 4 cm (deep) rockwool plugs that had been wetted using a feed solution with an electrical conductivity of 500 $\mu s\ cm^{-2}$ and a temperature of 25°C. This nutrient solution was obtained by dilution of the full-strength nutrient solution described. The rockwool plugs were maintained at 25°C under supplemental light at 160 W m^{-2} by using 400 W (high pressure sodium) lights placed in rows located 2 m above the seedlings, with 3m between lights in the rows, and 3.5 m between the rows of lights. The seedlings were misted with water for 1 minute, 3 times a day to maintain humidity, and were watered with the 500 µs cm^{-2} nutrient solution as required.

One week after sowing, when the first true leaves emerged, the seedlings were transplanted into 10 cm x 10 cm x 6.5 cm (deep) rockwool blocks containing a 4 cm diameter by 4 cm deep depression in the top of the block. Transplanting was accomplished by placing the rockwool plug containing the seedling into the depression in the top of the rockwool block. The rockwool blocks were thoroughly wetted with nutrient solution prior to transplanting, as described above. The young transplants were maintained under supplemental light as described above, with a root zone temperature of 23°C. The plants were watered with the 500 µs cm^{-2} nutrient solution as required.

At 5 weeks of age, when the plants had developed 8 true leaves, the plants were transplanted into the production greenhouse. The plants were placed on 20 L sawdust bags (spruce and pine mix, horticultural grade, Spray Lakes Sawmills), with 2 plants per bag arranged in 5 double rows of 12 bags per row. One drip tube was placed next to each plant to deliver the nutrient solution as required. The initial planting density was 2.0 plants m^{-2}, and when the plants were 12 weeks old, the density was increased to an effective 3.0 plants m^{-2} by allowing a lateral stem to develop into a second main stem on every second plant.

The plants were supported by twine tied to overhead wires located 3 m above the greenhouse floor, directly above the rows and running the length of the rows. The canopy was maintained at a height of 2.5 m. Pruning and training operations were completed twice a week. The plant structure was maintained to the selected main stems by pruning lateral stem growth. Flowers were pollinated by hand using battery powered "electric bee" pollinators to vibrate the flowering trusses every second day. Trusses were pruned to 4 or 5 fruit depending on the individual status of the plant.

The developing tomato fruit was harvested at the stage when the first yellowish-orange colouration was evident at the blossom end of the fruit. The fruit was graded according to commercial size standards Table A-1) into categories; extra large, large, medium, and small (Canada Agricultural Products Act, Fresh Fruits and Vegetables

Products Regulations). All cull fruit were also accounted for. Data was recorded on total weight in each size category and total yield in kg m^{-2}.

Control of the environment was obtained using an Argus® (Argus Control Systems Limited) environmental control system to manage the environment using a completely integrated approach. Air temperatures were managed to maintain a 24-hour mean temperature of 20°C, with warmer day temperatures to a maximum of 23°C and cooler night temperatures to a minimum of 18°C. Relative humidity was maintained between 70-75% using evaporative coolers to humidify and cool the greenhouse and heat and fans to dehumidify and heat the greenhouse. Root zone temperature was maintained at 20°C by circulating warm water, as required, through a network of pipes placed immediately underneath the sawdust bags.

The nutrient solution was applied to meet the daily water and fertilizer requirements of the plant by delivering the volume required at regular intervals during daylight hours. The requirements of the plants for the nutrient feed were determined by ensuring that a 15% over-drain target was maintained throughout the season. A monitoring station was established in the crop where the nutrient solution draining from the bottom of one sawdust bag (over-drain) was collected over the course of the day. The percent over-drain was calculated and when levels began to drop below 15%, the amount of nutrient solution delivered to the crop was increased. The EC of the over-drain was also monitored, and when the over-drain EC increased beyond 5500 $\mu s\ cm^{-2}$ the EC of the nutrient solution was lowered in order to maintain the over-drain EC below the maximum limit.

Carbon dioxide was supplemented to maintain a minimum level of 400 $\mu mol\ mol^{-1}$ using natural gas CO_2 generators in the first year of the trial. In the second year of the trial a liquid CO_2 supplementation system using a network of 5 cm diameter plastic pipe placed directly in the canopy 30 cm below the tops of the plants. One pipe was used for each double row. The change was made in the second year of the trial because the natural gas generators were not able to maintain the target CO_2 levels during the summer when ventilation rates were greater than 1 complete air exchange per minute. The CO_2 levels in the greenhouse under summer high light conditions and routinely fell to below

200 µmol mol^{-1}. The liquid CO_2 system was constructed in order to improve CO_2 use and increase production. The change in CO_2 supplementation systems was the only major change to the greenhouse system in the 2 years of the trial.

Insect pest control was accomplished entirely through the use of biological control agents in both years of the trial. The only insect pest that was noted in the crop was greenhouse whitefly (*Trialeuroides vapárariorum* Westwood) which was controlled using the parasitic wasp (*Encarsia formosa* Gahan).

Statistical analyses

Yield data were analyzed by an analysis of variance (ANOVA) using the general linear model (GLM) procedure (SAS Institute Inc.). The model used considered yield as a function of cultivar, which was considered a fixed factor. The treatment means were compared to the 5% level of significance using the Tukey test.

Results

In the first year of the trial harvesting began on May 17 and continued through to October 27 for a total of 22 weeks. The harvesting period in the second year was 30 weeks beginning on March 30 and ending on October 30. The yield in the first year of the trial was 28.88 and 32.41 kg m^{-2} for Rapsodie seed lots 1 and 2 respectively (Table A-2). The difference in yield between the 2 seed lots were significant. As the 2 cultivars were managed exactly the same, the differences inherent to distinct seed lots is the only explanation for the differences in yield. The yield in the second year of the trial was 59.23, 65.00, and 63.03 kg m^{-2} for cultivars Quest, Trust and Rapsodie respectively (Table A-3). There were no significant yield differences between the cultivars in the second year of the trial.

The average yield increase in the second year, over all cultivars was 2.04 times the yield in the first year. This represented an increase from a yield considered in the lower 20% of commercial yields, to a yield representing the to 5-10% of Alberta greenhouse tomato growers. The percentage of fruit by weight in the extra large and large categories was 82.71 and 81.82% for Rapsodie seed lots 1 and 2 respectively in year one. The percentage of fruit by weight in the extra large and large categories was 92.24, 93.88, and 91.84% for Quest, Trust, and Rapsodie respectively, in the second year of the trial. The percentage of cull fruit by weight for all cultivars was less than 1% in both years of the trial.

Discussion

The success of commercial tomato greenhouses is dependent on the efficient production of a high yielding, high quality crop. Tomato fruit yields exceeding 60 kg m^{-2} are considered excellent. In addition, commercial growers strive to maximize the number of fruit in the extra large and large categories and to minimize the number of unmarketable cull fruit. Typically growers will try to manage their crops so that the proportion of extra large and large fruit exceeds 80% of the total production with preferably no cull fruit to a maximum tolerable limit of 1%.

The fruit quality obtained in both years of the trial was acceptable by commercial standards and included a 10% increase in extra large and large fruit in the second year of the trial. In addition the yield in the second year was excellent, and represented what would be expected from a top yielding commercial greenhouse tomato operation in Alberta.

The reason for the yield increase in the second year of the trial can be partially explained by the increased skill due to the experience of the first year. However, the main reason was probably due to the use of the liquid CO_2 system as a clear improvement in the ability to maintain the CO_2 supplementation target of 400 μmol mol^{-1}, under the high summer ventilation rates, was seen in the second year. Yield increases in greenhouse tomatoes associated with providing increasing CO_2 levels are known to be significant. In British Columbia, an expected yield increase of 30% is associated with increasing CO_2 levels from 350 to 750 μmol mol^{-1} (Portree 1996).

Conclusions

This study provided a practical approach to develop a greenhouse tomato model system that is representative of a commercial tomato greenhouse. This system can be used to obtain a high yield of good quality tomatoes and can be used for the mass screening and development of strains of *T. harzianum* as biological control agents for *B. cinerea* in tomato. This greenhouse tomato production model system also presents an excellent opportunity for wider application of study in greenhouse tomato.

Tables and figures

Table A-1 Commercial grading size standards for tomatoes*

Category	Size (Diameter)
Extra Large	> 73 mm
Large	65–72 mm
Medium	51-64 mm
Small	< s50 mm

* According to the Canada Agricultural Products Act, Fresh Fruits and Vegetables Products Regulations.

Table A-2 **Yield and grade data from the 1998-1999 tomato production trial conducted with 2 different seed lots of Rapsodie.**

	Grade											
	X Large		Large		Medium		Small		Culls		Total	Mean Yield
Cultivar	Total No. of Fruit	Total Wt. (kg)	Total No. of Fruit	Total Wt. (kg)	Total No. of Fruit	Total Wt. (kg)	Total No. of Fruit	Total Wt. (kg)	Total No. of Fruit	Total Wt. (kg)	Weight (kg)	kg m-2
Rapsodie (Lot 1)												
Mean	197.50	48.98	260.00	46.55	129.75	17.08	24.25	2.10	3.75	0.80	115.50	28.88 a
Mean Fruit wt (kg)	0.25		0.18		0.13		0.09		0.21			
% of Total wt		42.41		40.30		14.79		1.82		0.68		
Rapsodie (Lot 2)												32.41 b
Mean	197.50	55.08	288.50	51.00	142.75	19.40	27.50	3.50	4.10	0.68	129.65	
Mean Fruit wt (kg)	0.28		0.18		0.14		0.13		0.16			
% of Total wt		42.48		39.34		14.96		2.70		0.52		

Note: Means followed by the same letter are not significantly different ($P \leq 0.05$) according to the Tukey test.

Table A-3 Yield and grade data from the 1998-1999 tomato production trial with cultivars Quest, Trust and Rapsodie.

	Grade											
Cultivar	**X Large**		**Large**		**Medium**		**Small**		**Culls**		**Total**	**Mean Yield**
	Total No. of Fruit	Total Wt. (kg)	Total No. of Fruit	Total Wt. (kg)	Total No. of Fruit	Total Wt. (kg)	Total No. of Fruit	Total Wt. (kg)	Total No. of Fruit	Total Wt. (kg)	**Weight (kg)**	**kg m-2**
Quest												
Mean	381.50	77.58	975.25	140.98	189.05	16.00	33.50	1.65	7.00	0.73	236.93	59.23 a
Mean Fruit wt. (kg)	0.20		0.14		0.08		0.05		0.10			
% of of Total wt. (kg)		32.74		59.50		6.75		0.70		0.31		
Trust												
Mean	446.75	94.63	1002.00	149.48	149.25	13.53	22.75	0.95	6.00	1.43	260.00	65.00 a
Mean Fruit wt. (kg)	0.21		0.15		0.09		0.04		0.24			
% of of Total wt. (kg)		36.39		57.49		5.20		0.37		0.55		
Rapsodie												
Mean	409.00	86.80	973.50	144.75	182.00	16.50	33.00	1.70	21.25	2.38	252.13	63.03 a
Mean Fruit wt. (kg)	0.21		0.15		0.09		0.05		0.11			
% of of Total wt. (kg)		34.43		57.41		6.54		0.67		0.94		

Note: Means followed by the same letter are not significantly different ($P \le 0.05$) according to the Tukey test.

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